IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Benjamin Eithan Reubinoff, et al.	**Examiner:**	Thaian N Ton
Serial No.:	09/808,382	**Art Unit:**	1632
Filed:	March 14, 2001	**Docket:**	14418
For:	EMBRYONIC STEM CELLS AND NEURAL PROGENITOR CELLS DERIVED THEREFROM		

Confirmation No: 1139

Commissioner for Patents
Alexandria, VA 22313-1450

DECLARATION OF Alan Colman UNDER 37 C.F.R. §1.132

Sir:

I, Alan Colman, hereby declare as follows:

1. I am Chief Executive Officer of ES Cell International Pte Ltd, 11 Biopolis Way, Helios Block #05-03/04-06, Singapore 138667.

2. I hold a Bachelor of Science (B.S.) Degree in Biochemistry and a Doctorate Degree in Molecular Biology.

3. A true and correct copy of my curriculum vitae is attached hereto as **Exhibit A**.

4. I have carefully reviewed the present patent application, Serial No. 09/808,382 ("the '382 application") and have read the Official Action dated November 21, 2005. I understand that the Examiner has cited Thomson et al. (Science 282: 1145-1147, 1998) and

Brustle et al. (Science 285: 754-756, 1999). It is the Examiner's contention that Thomson teaches human embryonic stem (hES) cells, and that Brustle teaches certain conditions for inducing differentiation of murine embryonic stem (mES) cells into glial precursors and for inducing differentiation of the glial precursors into oligodendrocytes. The Examiner is of the opinion that it would have been obvious for one skilled in the art to culture the hES cells of Thomson, under the conditions taught by Brustle for mES cells, to arrive at the present invention as claimed in claims 51, 95 and 99, with a reasonable expectation of success.

5. I have been asked to comment on the Examiner's position. In this regard, I have reviewed the Thomson reference and the Brustle reference, and am familiar with the contents of these references.

6. It is my opinion that there are significant differences between hES cells and mES cells, which were known to those skilled in the art at the time the priority document of the '382 application was filed on March 14, 2000, such that those skilled in the art would not have reasonably expected that the conditions developed with mES cells, as taught by Brustle, could be applied to hES cells to obtain human neural progenitor cells with success. The basis for my opinion is as follows.

7. The first successful isolation of hES cell lines was reported by Thomson et al. (Science 282: 1145-1147, 1998, **Exhibit B**), following his earlier work on generating ES lines from monkeys. In contrast, Evans and Kaufman generated the first mES lines in 1981 from mouse blastocysts by culturing inner cell mass (ICM) from pre-implantation embryos on feeder layers. Hence there was a period of delay of 17 years before hES cell lines were firstly isolated and cultured. The delay in successfully isolating and culturing the hES cells was due, at least in part, to difficulties in applying the mouse protocols to hES derivation.

8. It was clear from the first human ES line report (Thomson et al. 1998, **Exhibit B**) that there were differences between hES and mES lines. This report indicated that the presence of LIF could not prevent differentiation of hES, in contrast to the inhibitory effect of LIF on mES differentiation previously reported by Niwa et al. (Genes and Development 12: 2048 – 2060,

1998, **Exhibit C**). It was known in 1998 that LIF was one of a number of cytokines that could activate the JAK/STAT signaling pathway, as discussed in Niwa et al. (1998). Therefore, it was believed at the time that the JAK/STAT signaling pathway was in some way deficient in hES cells. This belief was subsequently confirmed by Ginis et al. (Developmental Biology 269: 360-380, 2004, **Exhibit D**), showing that GP130 and the LIF receptor, which together make up the LIF receptor complex, were missing in many hES lines.

9. Before 2000, the JAK/STAT signaling pathway had been implicated in many aspects of development including the division, survival and differentiation of embryonic central nervous system cells. See review by Cattaneo et al. (TINS 22: 365-369, 1999) (**Exhibit E**). Indeed, these authors reference the fact that LIF has been shown to be necessary for the development and survival of oligodendrocytes in an *in vitro* model. See page 367, right column, second paragraph of Cattaneo et al.

10. Therefore, it is my opinion that, given the understanding that hES but not mES cells lack a component of the JAK/STAT pathway, together with the importance of this pathway in the development of neural cells, there would have been no reasonable expectation, prior to March 14, 2000, that hES cells would behave in the same way as mES cells when subjected to neural differentiation protocols developed using mES cells.

11. Early reports published prior to 2000 also identified other differences between hES cells and mES cells. Thomson (1998, **Exhibit C**) investigating *in vitro* differentiation of hES cells reports only observations of lineages reminiscent of trophoblasts (expressing human chorionic gonadotrophin) and extra embryonic endoderm (alpha fetoprotein expressing). See page 1146, left column, third paragraph. In contrast, under similar *in vitro* culture conditions that allowed spontaneous differentiation, mES cells gave rise to the multiple lineages representative of all three embryonic germ layers. See, e.g., Doetschman et al. (J. Embryol Exp. Morphol. 87: 27-45 (1985) (**Exhibit F**).

12. Around the same time of the Thomson (1998) report, Svendsen and Smith provided a review (TINS 22: 357-364, 1999, **Exhibit G**), in which they posed the question "Can

human ES cells be grown in culture?" They discussed various methods for obtaining pluripotent cells from embryonic tissue. In the context of recognizing the similarities in the manner by which hES cells and mES cells are derived, the authors specifically state on page 360, 1st paragraph that:

> "However, caution is still required at this early stage of our understanding in this new area. It is not certain that human cells derived by either the blastocyst or germ cell route will show equivalent properties to mouse ES cells, ..."

13. Svendsen and Smith further discussed the differences in the proliferation capacity between human pluripotent stem cells (HPCs) and mES cells on page 360, 1st column:

> "The proliferative capacity of these HPC cultures is far from clear, but it appears that they are rather more difficult to expand than mouse ES cells. It is also uncertain as to whether these human cells use the same intracellular signaling pathways as mouse ES cells in order to sustain the self renewal cycle."

14. Referring to the hES results reported by Thomson et al. (1998), discussed in Paragraph 11 above, Svendsen and Smith stated:

> "Surprisingly, only extra-embryonic cell types were detected when differentiation of the blastocyst-derived cells was induced in vitro. This could simply be because appropriate permissive or inductive conditions have not been identified."

15. More recently, additional evidence showing the differences in differentiation behavior between mES and hES cells is reported by Xu et al. (Nature Biotechnology 20: 1261-1264, 2002, **Exhibit H**). Xu et al. employed the growth factor BMP4 to induce differentiation of hES cells to trophoblast. On the other hand, mES cells cannot form trophoblast, as discussed in Xu et al. in the bridging paragraph of page 1262-1263. Therefore, Xu et al. concluded: "These results underscore fundamental differences between human and mouse ES cells". See the abstract of this reference.

16. The differences between hES cells and mES cells are summarized in the following table:

Table 1: Differences between mES cell and hES cells

Mouse ES Cells	Human ES Cells
Easily cultured as separate cells	Extremely difficult to culture as single cells.
Do not differentiate to form trophoblast cells	Can differentiate to form trophoblast cells
Grow in attached rounded masses in which single cells are difficult to identify	Grow in flat colonies with distinct cell borders in monolayer cultures
Feeder cells can be replaced by LIF or related members of this cytokine family	Do not respond to LIF
LIF prevents differentiation of mES cells	LIF does not prevent differentiation of hES cells
LIF-activatable JAK/STAT pathway present in mES cells	LIF-activatable JAK/STAT pathway not present in hES cells
Can be cloned as single cells at a high frequency in the presence of LIF	Cannot be cloned as single cells

17. The infancy of the research of hES cells in 2000 carried with it many of the uncertainties that were evident in the research of the mES cells 20 years ago. It is my opinion that the differences between hES cells and mES cells were known to be significant in 2000 such that those skilled in the art would not be able to readily extrapolate the results achieved with mES cells to hES cells. Therefore, in my opinion, at the time the priority document of the '382 application was filed, those skilled in the art would not have had any reasonable expectation of success if those skilled in the art were to apply the differentiation conditions developed with mES cells, as taught by Brustle, to hES cells to obtain neural progenitor cells.

18. My opinion is also supported by Ginnis et al. (2004) (**Exhibit D**), referenced above. Ginnis et al. state on page 361, left column, middle paragraph:

> "[Ca]ution must be exercised in extrapolating the data that has accumulated on the properties of mouse ES cells to studies using

human ES cells, and further studies analysing the similarities and differences between mouse and human lines are required."

19. It is further observed that the Brustle reference teaches the derivation of glial precursors which can differentiate to oligodendrocytes and astrocytes only. In Brustle, no neurons were formed from the precursor cells, regardless of whether the precursors were cultured *in vitro* in the absence of growth factors (page 754, middle column, and Figure 1), or transplanted into rat spinal cords *in vivo* (page 754, last paragraph to page 755, first paragraph, and Figure 2). Brustle does not teach or demonstrate anywhere how to produce neural progenitor cells that are multipotent and can differentiate into neurons, oligodendrocytes and astrocytes, as produced by the methods of the '382 application. Therefore, it is my opinion that those skilled in the art not only would not have had a reasonable expectation of success in applying the conditions for culturing mouse ES cells, taught by Brustle, to human ES cells as discussed, but also would not have had a reasonable expectation of success in applying the conditions taught by Brustle to produce the multipotent human neural progenitor cells, as characterized in the '382 application.

20. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that those statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

By: Alan Colman Dated: 22nd March 2006

Curriculum Vitae

Name	Alan Colman	
Date of Birth	21st October 1948	
Current Address	156 Gibraltar Crescent, Singapore 759588	
Education	Robert Styring Scholar, The Queen's College, Oxford	1967 – 1971
	BA (Hons) Biochemistry, The Queen's College, Oxford	1967 – 1971
Career History	Ph.D. at MRC Laboratory of Molecular Biology Hills Road, Cambridge, UK Supervisor - Dr J B Gurdon FRS	1971 – 1974
	Demonstrator in Developmental Biology Zoology Department, Oxford University, UK	1974 – 1976
	Awarded Brown Junior Research Fellowship, Queens College, Oxford, UK	1976
	Lecturer in Department of Biological Sciences, University of Warwick, Coventry, UK	1976 – 1983
	Senior Lecturer, Department of Biological Sciences, University of Warwick, Coventry, UK	1983 – 1987
	Professor, Department of Biochemistry, University of Birmingham, UK	1987 – 1993
	Research Director, PPL Therapeutics plc Edinburgh, UK.(part-time)	1988 – 1993
	Research Director, PPL Therapeutics plc Edinburgh, UK (full-time)	1993 – 2002
	Chief Scientific Officer, ES Cell International Singapore	2002 – 2005
	Senior Scientist, Center for Molecular Medicine, Biomedical research Institute, Singapore	2004 –
	Adjunct Professor, Department of Biochemistry, National University of Singapore	2002 –
	Chief Executive Officer, ES Cell International Singapore	2005 –

Membership of Professional Bodies

British Biochemical Society

American Society of Cell Biology

European Molecular Biology Organization (elected 1989)

Membership of Editorial Boards

Editor, Seminars in Cell Biology	1989 – 2003
Trends in Biotechnology	1991 –

Membership of Grant Bodies

UK Wellcome Cell and Molecular Biology Board	1988 – 1991
UK Medical Research Council, Cell & Molecular Medicine Board	1992 – 1996
UK BBSRC Link Cell Engineering Steering Committee	1993 – 2000

Membership of Industrial Bodies

Chairman of the Science Advisory Committee of the UK BioIndustry Association	1994 – 1998

Other Responsibilities

Governor of The Babraham Institute, Cambridge I also sat on their Science Advisory Board and Audit Committee. The Babraham Institute is a highly regarded UK Government – Supported research institute with charitable status	1994 – 2001
Member of the International Advisory Board for the UK Centre for Tissue Engineering	2001 –
National Science and Technology Awards Committee (Singapore)	2003 –
Faculty Advisory Committee, Faculty of Science, National University of Singapore	2003 –
Member of the US-based International Society of Stem Cell Research's Industry and Governmental Affairs Committee	2005 –

Academic Research Interests & Experience

My PhD work concentrated on developing the use of the frog (Xenopus) oocyte and embryos for transcription of foreign DNA and in 1975 I published the first evidence that the oocyte machinery could be used in this way (ref 2). Subsequent work by my supervisor and others resulted in the first demonstration of the bone fide transcription, "ex vivo", of an eukaryotic gene.

The use of the frog oocyte and eggs to provide a surrogate test tube was an application which was to dominate my academic career. My primary interest was in using this system to understand generic mechanisms of protein secretion. The frog oocyte is a very large cell which is particularly amenable to a whole range of micromanipulative techniques, including microinjection. Using mRNA microinjection as a means to producing wild type or mutant and mammalian proteins, I was able to test a variety of "secretory" hypotheses prevalent at the time (ref 6).

This system also lent itself to some novel applications, particularly in the area of immunoglobulin assembly and secretion since it was possible to produce the various subunits at different times from each other, at different sites within the oocyte, and with different stoichiometries (ref 21). In two Nature papers in the early eighties, I was able to demonstrate the heterologous expression of a murine antibody in frog oocytes (ref 14), and also, for the first time, I demonstrated that antibodies were functional even as they were folded within the ER—an early demonstration of "intracellular immunisation" (ref 20)

As a result of the above work, I became fascinated with the prospect that the secretory mechanisms I was investigating, figured prominently in the early development of Xenopus, and a lot of my later work was involved in this area.

In order to pursue my objectives regarding protein secretion, I had to develop or refine various technical methods. Prominent amongst there was the use of injected antisense oligonucleotides to remove unwanted endogenous or foreign mRNA's. This resulted in one of the earliest (and most direct) demonstrations of the effectiveness and fidelity of the antisense oligonucleotide methods (ref 48).

Commercial Research Activities and Responsibilities

1 PPL Therapeutics (1987-2002)

Research

During my academic career, I became interested in the commercial exploitation of the biomedical sciences and acted as a consultant to a variety of companies and financial houses, in the period 1981-1987 (e.g. ICI, Amersham, Rothschilds, Prudential, Transatlantic Capital). In 1987, I was invited to be a member of the Science Advisory Board of PPL Therapeutics at its inception that year. The initial mission of this company was to produce therapeutic proteins in the milk of transgenic livestock animals, an endeavour which meshed well with my previous experience with protein secretion. In 1988, as PPL's part-time Research Director, I recruited the first scientists and embryologists to the Company. I became full time Research Director in 1993 (leaving Birmingham University). During the period 1988 - present I was responsible for guiding and implementing PPL's strategic research programme. During this period, PPL grew to around 270 employees with approximately 120 in research.

Research highlights of this period were :

- Birth of Tracy, the highest producer of human protein in the milk of a transgenic mammal. (ref 67). She now resides (stuffed) in the Natural Science Museum in London.
- First report of the complete removal and replacement of a mouse gene with its human homologue. (refs 72,77)
- Establishment of the infrastructure (at PPL Inc, USA) and recruitment of appropriate personnel which has been responsible for the production of the largest number of transgenic founder bovines by any one company.
- Development of a generic method for deriving mammalian embryonic stem cell lines. (ref 83)
- Birth of Dolly (in collaboration with the Roslin Institute) the world's first mammal cloned from an adult cell.
- Birth of Polly (with Roslin Institute), the world's first transgenic, cloned mammal. (ref 85)
- In charge of very successful programme in gene targeting in primary somatic cells which resulted in the births of Diana and Cupid, the world's first livestock with targeted genetic changes (ref 95). I am a named inventor on the patent protecting this work.
- In charge of programme which led to the birth of the world's first cloned pigs. (ref 98)
- Lead the program which recently resulted in production of cloned alpha gal knock out pigs. (Ref 100)
- Developed innovative transgenic and bacterial technology for large scale, cost-effective production of modified, biologically active peptides. (101)
- I was responsible for developing PPL's focus on the use of embryonic stem cells to provide transplantable islets for patients suffering from diabetes. I am a named inventor on a patent covering a method of making ES cells without the need to create and destroy a mammalian embryo.
- Awarded three LINK awards (from three applications) by the UK government and 2 ATP (1999 and 2000) awards from US National Institute of Science and Technology.

As Research Director in charge of over 100 people, I established line management, project management, and a variety of reporting structures within PPL. Before Nov 1999, I had overall responsibility for a research budget of approximately £5-6 million, per annum. In Nov 1999, PPL had to downsize and Research was hard hit. When I left PPL the research team numbered around 50+people.

Product development

Although I had no direct responsibilities for Development, I participated in all the management discussions in getting products to the market and am aware of all the main issues.

Patents

PPL's Patent Department reported to me, and I am knowledgeable about patenting issues and strategies both in the US and Europe.

Collaborations

I was responsible for identifying and negotiating many academic collaborations.

Corporate matters

From 1987 – 1999, PPL was a privately owned company funded mainly by venture capital. In 1996, the company floated on the London Stock Exchange. As an executive director, I was fully involved in all these rounds of funding, receiving a broad education on corporate finance in the process.

2 ES Cell International 2002 –

I resigned from PPL on Mar 31 2002 in order to pursue my interests in human embryonic stem cells (hES) with ES Cell International (ESI) where I am now the Chief Executive Officer (CEO). I was Chief Scientific Officer (CSO) from April 2002–Feb 2005. ESI is now concentrating on developing clinically acceptable hES lines as well as devising methods of converting them into either pancreatic beta cells or beating cardiomyocytes in order to address the diabetes and congestive heart failure markets, respectively. As of November 2005, the company has 41 employees. Currently involved in raising equity funds.

3 Senior Scientist, Centre for Molecular Medicine 2004–

Seconded to the Singapore Government's A*STAR Centre for Molecular Medicine for 20% time to recruit, mentor and lead 10+ scientists in the Regenerative Medicine section.

Other activities and skills

Presentational skills

I get the opportunity to make countless presentations (many through invitation) at a wide variety of academic, industrial and financial venues. It is an activity I believe I am very good at.

International Meeting Organisation (recent)

Co-organiser of International Stem Cell Meeting in Singapore Oct 2003
Co-organiser of Keystone "Stem Cells, Senescence and Cancer" meeting in Singapore Oct 2005
Co-organiser of Regerarate 2006 in Pittsburg, USA

Science and Society

I have often been invited (particularly by EMBO) to act as a spokesperson at meetings on bioethical issues related to cloning, stem cells, and the general use of animals in research . I contributed one article to the new EMBO Reports on the recent controversy regarding the generation and use of human ES cells (99) and another regarding the interface between philosophy and genetic science. (103)

References (Can be supplied on request)

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Publications

1. Colman A, (1974) synthesis of RNA in oocytes of Xenopus laevis during culture in vitro. J. Embryol. Exp.Morph. 32 : 515-532.

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Embryonic Stem Cell Lines Derived from Human Blastocysts

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Human blastocyst-derived, pluripotent cell lines are described that have normal karyotypes, express high levels of telomerase activity, and express cell surface markers that characterize primate embryonic stem cells but do not characterize other early lineages. After undifferentiated proliferation in vitro for 4 to 5 months, these cells still maintained the developmental potential to form trophoblast and derivatives of all three embryonic germ layers, including gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm). These cell lines should be useful in human developmental biology, drug discovery, and transplantation medicine.

Embryonic stem (ES) cells are derived from totipotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation in vitro (*1*, *2*). In chimeras with intact embryos, mouse ES cells contribute to a wide range of adult tissues, including germ cells, providing a powerful approach for introducing specific genetic changes into the mouse germ line (*3*). The term "ES cell" was introduced to distinguish these embryo-derived pluripotent cells from teratocarcinoma-derived pluripotent embryonal carcinoma (EC) cells (*2*). Given the historical introduction of the term "ES cell" and the properties of mouse ES cells, we proposed that the essential characteristics of primate ES cells should include (i) derivation from the preimplantation or periimplantation embryo, (ii) prolonged undifferentiated proliferation, and (iii) stable developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture (*4*). For ethical and practical reasons, in many primate species, including humans, the ability of ES cells to contribute to the germ line in chimeras is not a testable property. Nonhuman primate ES cell lines provide an accurate in vitro model for understanding the differentiation of human tissues (*4*, *5*). We now describe human cell lines that fulfill our proposed criteria to define primate ES cells.

Fresh or frozen cleavage stage human embryos, produced by in vitro fertilization (IVF) for clinical purposes, were donated by individuals after informed consent and after institutional review board approval. Embryos were cultured to the blastocyst stage, 14 inner cell masses were isolated, and five ES cell lines originating from five separate embryos were derived, essentially as described for nonhuman primate ES cells (*5*, *6*). The resulting cells had a high ratio of nucleus to cytoplasm, prominent nucleoli, and a colony morphology similar to that of rhesus monkey ES cells (Fig. 1). Three cell lines (H1, H13, and H14) had a normal XY karyotype, and two cell lines (H7 and H9) had a normal XX karyotype. Each of the cell lines was successfully cryopreserved and thawed. Four of the cell lines were cryopreserved after 5 to 6 months of continuous undifferentiated proliferation. The other cell line, H9, retained a normal XX karyotype after 6 months of culture and has now been passaged continuously for more than 8 months (32 passages). A period of replicative crisis was not observed for any of the cell lines.

The human ES cell lines expressed high levels of telomerase activity (Fig. 2). Telomerase is a ribonucleoprotein that adds telomere repeats to chromosome ends and is involved in maintaining telomere length, which plays an important role in replicative life-span (*7*, *8*). Telomerase expression is highly correlated with immortality in human cell lines, and reintroduction of telomerase activity into some diploid human somatic cell lines extends replicative life-span (*9*). Diploid human somatic cells do not express telomerase, have shortened telomeres with age, and enter replicative senescence after a finite proliferative life-span in tissue culture (*10*–*13*). In contrast, telomerase is present at high levels in germ line and embryonic tissues (*14*). The high level of telomerase activity expressed by the human ES cell lines therefore suggests that their replicative life-span will exceed that of somatic cells.

The human ES cell lines expressed cell surface markers that characterize undifferentiated nonhuman primate ES and human EC cells, including stage-specific embryonic antigen (SSEA)–3, SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase (Fig. 3) (*4*, *5*, *15*, *16*). The globo-series glycolipid GL7, which carries the SSEA-4 epitope, is formed by the addition of sialic acid to the globo-series glycolipid Gb5, which carries the SSEA-3 epitope (*17*, *18*). Thus, GL7 reacts with antibodies to both SSEA-3 and SSEA-4 (*17*, *18*). Staining intensity for SSEA-4 on the human ES cell lines was consistently strong, but staining intensity for SSEA-3 was weak and varied both within and among colonies (Fig. 3, D and C). Because GL7 carries both the SSEA-4 and SSEA-3 epitopes and because staining for SSEA-4 was consistently strong, the relatively weak staining for

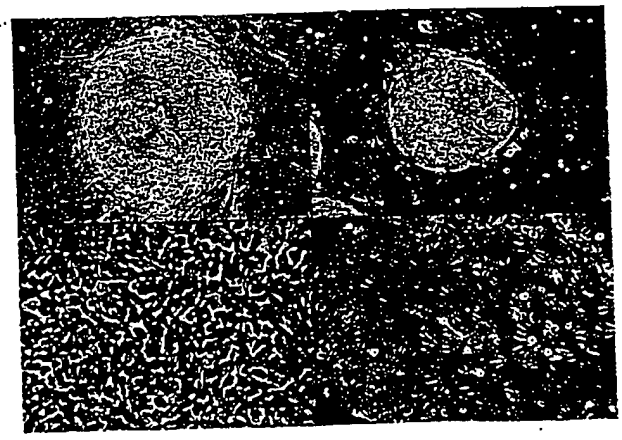

Fig. 1. Derivation of the H9 cell line. (A) Inner cell mass–derived cells attached to mouse embryonic fibroblast feeder layer after 8 days of culture, 24 hours before first dissociation. Scale bar, 100 μm. (B) H9 colony. Scale bar, 100 μm. (C) H9 cells. Scale bar, 50 μm. (D) Differentiated H9 cells, cultured for 5 days in the absence of mouse embryonic fibroblasts, but in the presence of human LIF (20 ng/ml; Sigma). Scale bar, 100 μm.

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SSEA-3 suggests a restricted access of the antibody to the SSEA-3 epitope. In common with human EC cells, the undifferentiated human ES cell lines did not stain for SSEA-1, but differentiated cells stained strongly for SSEA-1 (*15*) (Fig. 3). Mouse inner cell mass cells, ES cells, and EC cells express SSEA-1 but do not express SSEA-3 or SSEA-4 (*17*, *19*), suggesting basic species differences between early mouse and human development.

The human ES cell lines were derived by the selection and expansion of individual colonies of a uniform, undifferentiated morphology, but none of the ES cell lines was derived by the clonal expansion of a single cell. The uniform undifferentiated morphology that is shared by human ES and nonhuman primate ES cells and the consistent expression by the human ES cell lines of cell surface markers that uniquely characterize primate ES and human EC cells make it extremely unlikely that a mixed population of precursor cells was expanded. However, because the cell lines were not cloned from a single cell, we cannot rule out the possibility that there is some variation in developmental potential among the undifferentiated cells, in spite of their homogeneous appearance.

The human ES cell lines maintained the potential to form derivatives of all three embryonic germ layers. All five cell lines produced teratomas after injection into severe combined immunodeficient (SCID)–beige mice. Each injected mouse formed a teratoma, and all teratomas included gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm) (Fig. 4). In vitro, the ES cells differentiated when cultured in the absence of mouse embryonic fibroblast feeder layers, both in the presence and absence of human leukemia inhibitory factor (LIF) (Fig. 1). When grown to confluence and allowed to pile up in the culture dish, the ES cell lines differentiated spontaneously even in the presence of fibroblasts. After H9 cells were allowed to differentiate for 2 weeks, both α-fetoprotein (350.9 ± 14.2 IU/ml) and human chorionic gonadotropin (hCG, 46.7 ± 5.6 mIU/ml) were detected in conditioned culture medium, indicating endoderm and trophoblast differentiation (*20*).

Human ES cells should offer insights into developmental events that cannot be studied directly in the intact human embryo but that have important consequences in clinical areas, including birth defects, infertility, and pregnancy loss. Particularly in the early postimplantation period, knowledge of normal human development is largely restricted to the description of a limited number of sectioned embryos and to analogies drawn from the experimental embryology of other species (*21*). Although the mouse is the mainstay of experimental mammalian embryology, early structures including the placenta, extraembryonic membranes, and the egg cylinder all differ substantially from the corresponding structure of the human embryo. Human ES cells will be particularly valuable for the study of the development and function of tissues that differ between mice and humans: Screens based on the in vitro differentiation of human ES cells to specific lineages could identify gene targets for new drugs, genes that could be used for tissue regeneration therapies, and teratogenic or toxic compounds.

Elucidating the mechanisms that control differentiation will facilitate the efficient, directed differentiation of ES cells to specific cell types. The standardized production of large, purified populations of euploid human cells such as cardiomyocytes and neurons will provide a potentially limitless source of cells for drug discovery and

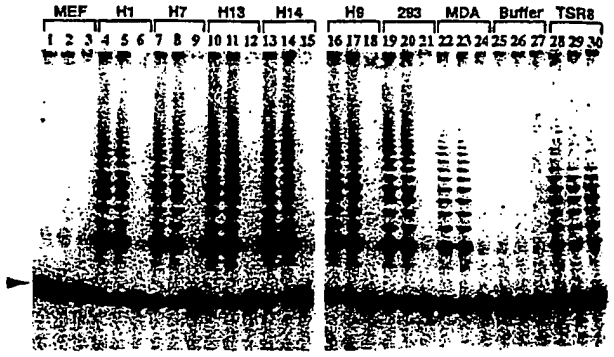

Fig. 2. Telomerase expression by human ES cell lines. MEF, irradiated mouse embryonic fibroblasts used as a feeder layer for the cells in lanes 4 to 18; 293, adenovirus-transformed kidney epithelial cell line 293; MDA, breast cancer cell line MDA; TSR8, quantitation control template. Telomerase activity was measured with the TRAPEZE Telomerase Detection Kit (Oncor, Gaithersburg, Maryland). The ES cell lines were analyzed at passages 10 to 13. About 2000 cells were assayed for each telomeric repeat amplification protocol assay, and 800 cell equivalents were loaded in each well of a 12.5% nondenaturing polyacrylamide gel. Reactions were done in triplicate with the third sample of each triplet heat inactivated for 10 to 15 min at 85°C before reaction to test for telomerase heat sensitivity (lanes 6, 9, 12, 15, 18, 21, 24, and 27). A 36–base pair internal control for amplification efficiency and quantitative analysis was run for each reaction as indicated by the arrowhead. Data were analyzed with the Storm 840 Scanner and ImageQuant package (Molecular Dynamics). Telomerase activity in the human ES cell lines ranged from 3.8 to 5.9 times that observed in the immortal human cell line MDA on a per cell basis.

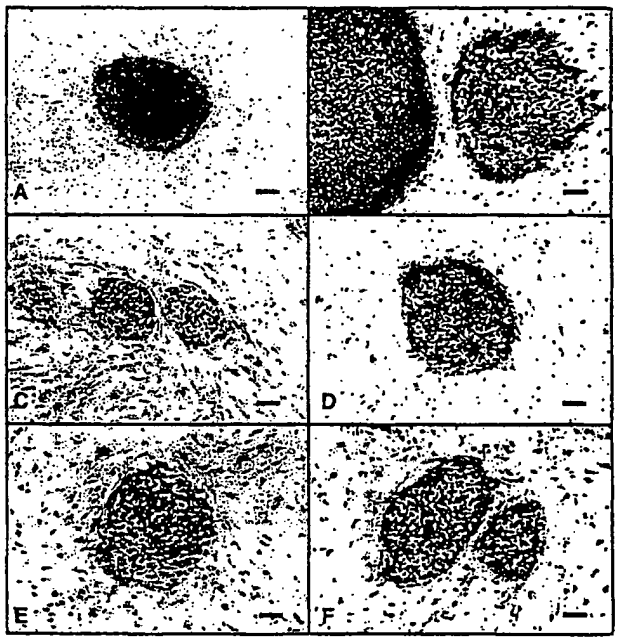

Fig. 3. Expression of cell surface markers by H9 cells. Scale bar, 100 μm. (A) Alkaline phosphatase. (B) SSEA-1. Undifferentiated cells failed to stain for SSEA-1 (large colony, left). Occasional colonies consisted of nonstained, central, undifferentiated cells surrounded by a margin of stained, differentiated, epithelial cells (small colony, right). (C) SSEA-3. Some small colonies stained uniformly for SSEA-3 (colony left of center), but most colonies contained a mixture of weakly stained cells and a majority of nonstained cells (colony right of center). (D) SSEA-4. (E) TRA-1-60. (F) TRA-1-81. Similar results were obtained for cell lines H1, H7, H13, and H14.

Fig. 4. Teratomas formed by the human ES cell lines in SCID-beige mice. Human ES cells after 4 to 5 months of culture (passages 14 to 16) from about 50% confluent six-well plates were injected into the rear leg muscles of 4-week-old male SCID-beige mice (two or more mice per cell line). Seven to eight weeks after injection, the resulting teratomas were examined histologically. (A) Gutlike structures. Cell line H9. Scale bar, 400 μm. (B) Rosettes of neural epithelium. Cell line H14. Scale bar, 200 μm. (C) Bone. Cell line H14. Scale bar, 100 μm. (D) Cartilage. Cell line H9. Scale bar, 100 μm. (E) Striated muscle. Cell line H13. Scale bar, 25 μm. (F) Tubules interspersed with structures resembling fetal glomeruli. Cell line H9. Scale bar, 100 μm.

transplantation therapies. Many diseases, such as Parkinson's disease and juvenile-onset diabetes mellitus, result from the death or dysfunction of just one or a few cell types. The replacement of those cells could offer lifelong treatment. Strategies to prevent immune rejection of the transplanted cells need to be developed but could include banking ES cells with defined major histocompatibility complex backgrounds or genetically manipulating ES cells to reduce or actively combat immune rejection. Because of the similarities to humans and human ES cells, rhesus monkeys and rhesus ES cells provide an accurate model for developing strategies to prevent immune rejection of transplanted cells and for demonstrating the safety and efficacy of ES cell–based therapies. Substantial advances in basic developmental biology are required to direct ES cells efficiently to lineages of human clinical importance. However, progress has already been made in the in vitro differentiation of mouse ES cells to neurons, hematopoietic cells, and cardiac muscle (*22–24*). Progress in basic developmental biology is now extremely rapid; human ES cells will link this progress even more closely to the prevention and treatment of human disease.

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6. Thirty-six fresh or frozen-thawed donated human embryos produced by IVF were cultured to the blastocyst stage in G1.2 and G2.2 medium (*25*). Fourteen of the 20 blastocysts that developed were selected for ES cell isolation, as described for rhesus monkey ES cells (*5*). The inner cell masses were isolated by immunosurgery (*26*), with a rabbit antiserum to BeWO cells, and plated on irradiated (35 grays gamma irradiation) mouse embryonic fibroblasts. Culture medium consisted of 80% Dulbecco's modified Eagle's medium (no pyruvate, high glucose formulation; Gibco-BRL) supplemented with 20% fetal bovine serum (Hyclone), 1 mM glutamine, 0.1 mM β-mercaptoethanol (Sigma), and 1% nonessential amino acid stock (Gibco-BRL). After 9 to 15 days, inner cell mass–derived outgrowths were dissociated into clumps either by exposure to Ca^{2+}/Mg^{2+}-free phosphate-buffered saline with 1 mM EDTA (cell line H1), by exposure to dispase (10 mg/ml; Sigma; cell line H7), or by mechanical dissociation with a micropipette (cell lines H9, H13, and H14) and replated on irradiated mouse embryonic fibroblasts in fresh medium. Individual colonies with a uniform undifferentiated morphology were individually selected by micropipette, mechanically dissociated into clumps, and replated. Once established and expanded, cultures were passaged by exposure to type IV collagenase (1 mg/ml; Gibco-BRL) or by selection of individual colonies by micropipette. Clump sizes of about 50 to 100 cells were optimal. Cell lines were initially karyotyped at passages 2 to 7.
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20. hCG and α-fetoprotein were measured by specific radioimmunoassay (double AB hCG and AFP-TC kits; Diagnostic Products, Los Angeles, CA). hCG assays used the World Health Organization Third International Standard 75/537. H9 cells were allowed to grow to confluence (day 0) on plates of irradiated mouse embryonic-fibroblasts. Medium was replaced daily. After 2 weeks of differentiation, medium in triplicate wells conditioned for 24 hours was assayed for hCG and α-fetoprotein. No hCG or α-fetoprotein was detected in unconditioned medium.
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Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3

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The propagation of embryonic stem (ES) cells in an undifferentiated pluripotent state is dependent on leukemia inhibitory factor (LIF) or related cytokines. These factors act through receptor complexes containing the signal transducer gp130. The downstream mechanisms that lead to ES cell self-renewal have not been delineated, however. In this study, chimeric receptors were introduced into ES cells. Biochemical and functional studies of transfected cells demonstrated a requirement for engagement and activation of the latent trancription factor STAT3. Detailed mutational analyses unexpectedly revealed that the four STAT3 docking sites in gp130 are not functionally equivalent. The role of STAT3 was then investigated using the dominant interfering mutant, STAT3F. ES cells that expressed this molecule constitutively could not be isolated. An episomal supertransfection strategy was therefore used to enable the consequences of STAT3F expression to be examined. In addition, an inducible STAT3F transgene was generated. In both cases, expression of STAT3F in ES cells growing in the presence of LIF specifically abrogated self-renewal and promoted differentiation. These complementary approaches establish that STAT3 plays a central role in the maintenance of the pluripotential stem cell phenotype. This contrasts with the involvement of STAT3 in the induction of differentiation in somatic cell types. Cell type-specific interpretation of STAT3 activation thus appears to be pivotal to the diverse developmental effects of the LIF family of cytokines. Identification of STAT3 as a key transcriptional determinant of ES cell self-renewal represents a first step in the molecular characterization of pluripotency.

[*Key Words:* Leukemia inhibitory factor (LIF); cytokine receptor; signaling; ES cells; tetracycline; episome]

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Embryonic stem (ES) cells are pluripotent cell lines derived by culture of preimplantation mouse embryos (Evans and Kaufman 1981; Martin 1981; Brook and Gardner 1997). At present, ES cells are the only nontransformed mammalian stem cells that can be continuously propagated in vitro. ES cell self-renewal is sustained by the cytokine leukemia inhibitory factor (LIF) (Smith and Hooper 1987; Smith et al. 1988; Williams et al. 1988). The effect of LIF is to inhibit differentiation and support proliferation of undifferentiated stem cells. However, the mechanisms underlying the maintenance of pluripotency during proliferative expansion remain elusive. We are attempting to define those signaling processes downstream of the LIF receptor complex that direct ES cell self-renewal. Elucidation of these principles will provide a molecular model for stem cell regulation in mammals. Insights provided by such a model should also be directly applicable to the extension of ES cell technology to non-mouse species.

The actions of LIF are mediated via heterodimerization of two members of the class I cytokine receptors, the low-affinity LIF receptor (LIF-R) and gp130 (Gearing et al. 1991; Gearing and Bruce 1992; Davis et al. 1993). The LIF-related cytokines, oncostatin M (OSM), cardiotrophin (CT-1), and ciliary neurotrophic factor (CNTF), act through the same receptor complex (in the case of CNTF, additionally including the CNTF-Rα subunit) and can similarly sustain ES cell self-renewal (Conover et al. 1993; Rose et al. 1994; Wolf et al. 1994; Yoshida et al. 1994; Pennica et al. 1995b). Furthermore, ES cells can also be derived and maintained using a combination of interleukin-6 and soluble interleukin-6 receptor (IL-6/sIL-6R) (Nichols et al. 1994; Yoshida et al. 1994). In this case, signaling is initiated via formation of gp130 homodimers without involvement of LIF-R (Murakami et al. 1993; Yoshida et al. 1994). Signals that emanate from gp130 are therefore sufficient for self-renewal.

gp130 mediates cellular responses to IL-6 and IL-11 in addition to the LIF-related cytokines (Kishimoto et al. 1994). All of these factors exert pleiotropic effects on

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diverse cell types in vitro and in vivo. In addition to ES cell self-renewal, stimulation of gp130 receptor complexes causes differentiation and growth inhibition in M1 myeloid leukemic cells (Tomida et al. 1984), induction of acute phase gene expression in hepatocytes (Baumann and Wong 1989), cholinergic differentiation of sympathetic neurons (Yamamori et al. 1989), survival of motor neurons (Li et al. 1995), proliferative and hypertrophic responses in cardiomyocytes (Hirota et al. 1995; Pennica et al. 1995a; Yoshida et al. 1996), and astrocyte differentiation of neuroepithelial progenitors (Bonni et al. 1997; Koblar et al. 1998).

Signaling processes downstream of gp130 are complex and are not yet fully characterized. Ligand-induced dimerization of the receptors (Davis et al. 1993; Murakami et al. 1993) leads to phosphorylation and activation of associated JAK tyrosine kinases (Narazaki et al. 1994; Stahl et al. 1994). The cytoplasmic domain of gp130 contains several tyrosine residues that are phosphorylated by the activated JAKs. These phosphotyrosine residues then interact with SH2 domain containing proteins that in turn themselves become targets for JAKs and possibly other nonreceptor tyrosine kinases. Consequences include activation of the Ras mitogen-activated protein (MAP) kinase (ERK) signaling cascade (Boulton et al. 1994; Yin and Yang 1994; Sheng et al. 1997) and of the STAT factors STAT1 and STAT3 (Lutticken et al. 1994; Stahl et al. 1995). STAT proteins are latent transcription factors that upon phosphorylation, dimerize and translocate to the nucleus where they activate target gene transcription (for review, see Ihle 1996). In myeloid leukemic M1 cells, activation of STAT3 appears to be the main effector of the differentiation response to IL-6 or LIF (Minami et al. 1996; Nakajima et al. 1996). STAT3 activation has also been adduced to mediate CNTF or LIF-induced differentiation of neuroepithelial precursors into astrocytes (Bonni et al. 1997).

In this study we have examined the receptor requirements for self-renewal signaling in ES cells and determined a critical contribution of STAT3 activation. In contrast to its role in somatic cells, activated STAT3 acts to suppress differentiation in ES cells.

Results

Granulocyte colony-stimulating factor receptor can signal ES cell self-renewal

Granulocyte colony-stimulating factor receptor (G-CSF-R) is a class I cytokine receptor that is evolutionarily related to gp130 and LIF-R (Gearing et al. 1991; Chambers et al. 1997). G-CSF-R is not present in ES cells. To begin delineating the signaling requirements for ES cell propagation, the capacity of these related receptors to sustain self-renewal was compared directly.

G-CSF-R undergoes ligand-induced homodimerization to produce an active signaling complex. G-CSF responsiveness can therefore be conferred on cytoplasmic domains of heterologous receptors through construction of appropriate fusions. cDNAs encoding full-length G-CSF-R cDNA and fusions between the extracellular portion of G-CSF-R and the transmembrane and cytoplasmic region of gp130 or LIF-R were cloned into the expression vector pPCAGIZ. Plasmids were introduced into LIF-R-deficient ES cells to eliminate the contribution of autocrine LIF signaling (Rathjen et al. 1990) from subsequent analyses. In this and all other experiments, ES cells were grown without feeder layers (Smith 1991). Transfectants were selected and expanded in the presence of IL-6/sIL-6R, acting through endogenous gp130, to avoid any selective pressure for adaptation to the introduced receptor.

Stable transfectants were then plated at clonal density in the absence of cytokine or presence of IL-6/sIL-6R or G-CSF. The number of stem cell colonies generated was scored after 6 days. The data in Figure 1A show that the G-CSF-R/gp130 chimeric receptor sustained stem cell propagation in response to G-CSF. This result is an-

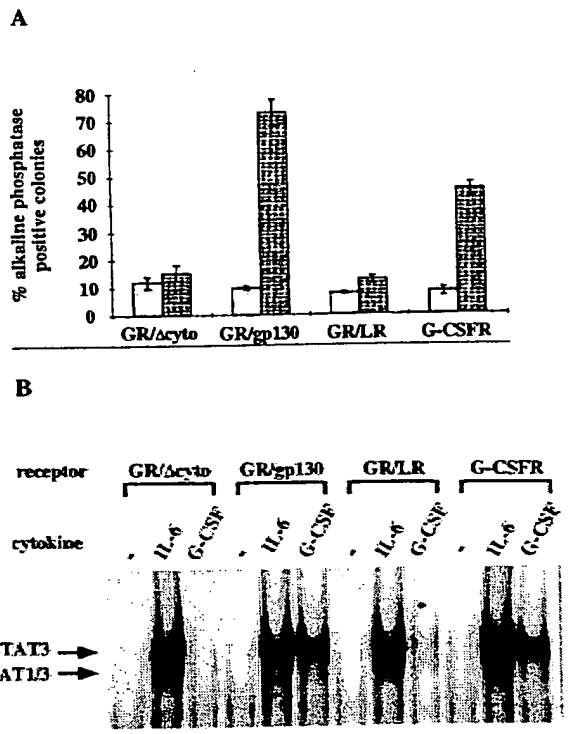

Figure 1. ES cell self-renewal and induction of STAT DNA-binding activity mediated by G-CSF-R wild-type, truncated, and chimeric cytokine receptors. (*A*) Efficiency of clonal stem cell renewal in response to G-CSF measured by formation of alkaline phosphatase-positive colonies. (Light gray bars) –G-CSF; (dark gray bars) +G-CSF. Data are mean ± S.E.M. of triplicate determinations on single representative clones normalized to response to IL-6/sIL-6R. (*B*) Induction of STAT DNA binding by IL-6/sIL-6R and G-CSF determined by electophoretic mobility-shift assay. Cells were untreated or stimulated for 30 min with IL-6/sIL-6R or G-CSF (30 ng/ml). Nuclear extracts were prepared and assayed for SIE binding. Note the absence of detectable STAT1/STAT3 heterodimer complex on stimulation of full-length G-CSF-R.

ticipated from previous findings on the capacity of gp130 homodimers to signal self-renewal (Yoshida et al. 1994). The G-CSF-R/LIF-R chimera did not support formation of stem cell colonies despite higher levels of cell surface expression measured by radioligand binding (not shown). This is in line with previous reports that homodimerization of the LIF-R cytoplasmic domain results in quantitatively (Baumann et al. 1994a; Stahl et al. 1995) and qualitatively (Stahl et al. 1995) diminished activation of downstream pathways compared with LIF-R/gp130 heterodimerization or gp130 homodimerization. However, ES cells transfected with G-CSF-R did form stem cell colonies in response to G-CSF-R though with lower efficiency than cells expressing the G-CSF-R/gp130 chimera. This somewhat surprising finding corroborates similar data reported recently (Starr et al. 1997). Propagation of the G-CSF-R transfectants remained factor dependent, and the cells differentiated normally when deprived of cytokine.

The finding that G-CSF-R is competent to maintain the stem cell phenotype suggests that the signaling interactions essential for ES cell self-renewal are preserved between gp130 and G-CSF-R. Conserved features in the intracellular domains of these two receptors are not readily identifiable because of extensive sequence divergence. However, G-CSF-R contains a putative STAT binding site and is thought to signal primarily through activation of STAT3 (Shimozaki et al. 1997). Electrophoretic mobility-shift assays were performed to determine the induction of nuclear STAT DNA-binding activity by G-CSF in the various ES cell transfectants. Significant STAT3 activation was evident in ES cells transfected with expression vectors for the G-CSF-R/gp130 chimera or the full-length G-CSF-R. In contrast, ES cells expressing the G-CSF-R/LIF-R chimera showed only weak induction of STAT3 DNA-binding activity in response to G-CSF (Fig. 1B). Antibody supershift experiments (not shown) confirmed that the DNA-binding complex consisted predominantly of STAT3 homodimers with a minor component of STAT3/STAT1 heterodimer as described previously in ES cells and other systems (Hocke et al. 1995; Stahl et al. 1995; Starr et al. 1997). These observations pointed to a potentially critical role for STAT3 activation in mediation of the self-renewal signal.

STAT3 docking sites on gp130 are required to signal ES cell self-renewal

The cytoplasmic domain of mouse gp130 contains seven tyrosine residues. Four of these have been identified as phosphorylation-dependent sites of interaction with STAT3 (Stahl et al. 1995). Substitution of these tyrosine residues with phenylalanine in the context of the G-CSF-R/gp130 chimera was therefore used to determine their significance for self-renewal signaling. The modified chimeric receptor expression constructs were introduced into DO27 ES cells. These cells are LIF-deficient because of targeted deletion of both gene copies and, in addition, carry a β-galactosidase reporter integrated into one allele of the *Oct-4* gene (C. Dani, I. Chambers, S. Johnstone, M. Robertson, B. Ebrahimi-Chahardahcherik, M. Saito, T. Taga, M. Li, T. Burdon, J. Nichols, and A.G. Smith, in prep.). This reporter is expressed only in undifferentiated ES cells (Mountford et al. 1994). Self-renewal was assayed both by measuring β-galactosidase activity in medium density cultures (Fig. 2B) and by scoring formation of alkaline phosphatase positive colonies at clonal density (Fig. 2C). Three independent transfectant clones were analyzed for each receptor. The data summarized in Figure 2 demonstrate that the presence of STAT3 docking sites is essential for stem cell propagation.

The intact gp130 cytoplasmic domain mediated a clear induction of SIE DNA-binding activity (Fig. 2D). Mutation of individual docking sites had no appreciable effect. However, mutation of all four sites eliminated both the self-renewal signal and the induction of STAT3 DNA-binding activity. Radioligand binding established that cell surface expression was not limiting for any of the receptors (not shown). To confirm that other signaling pathways are not impaired by mutation of the STAT3 docking sites, we examined activation of the ERK cascade. ERK activation requires receptor phosphorylation on tyrosine 118 by JAK kinases and recruitment of SHP2 (Stahl et al. 1995; Fukada et al. 1996). Figure 2E shows that the basal level of constitutive ERK activity was significantly enhanced by stimulation of chimeric receptors in all transfectants tested. In particular, the two receptors, Y265/275F and Y126-275F, which gave reduced activation of STAT3 and cannot signal self-renewal, mediated normal and heightened levels of ERK activation, respectively. Therefore, there is no general compromise in the signaling capacity of these molecules.

Interestingly, this data also indicates that the STAT3 sites in gp130 may not be equivalent in vivo. Specifically, mutation of the two adjacent carboxy-terminal STAT3 binding sites (Y265 and Y275) abolished self-renewal signaling, whereas mutation of the two-membrane proximal sites had little effect. This difference correlated with the lower induction of STAT3 DNA-binding activity and the specific reduction in STAT3 phosphorylation relative to ERK phosphorylation (Figs. 2D,E) (see Discussion). Self-renewal thus appears to require an appreciable level of STAT3 activation.

Inhibition of STAT3 activation blocks self-renewal and promotes differentiation

The above findings indicated that STAT3 may play a key role in ES cell signaling. To assess directly the requirement for STAT3 activation in ES cell self-renewal, we exploited a dominant interfering mutant form of STAT3, STAT3F. In this mutant (Minami et al. 1996), the tyrosine residue at amino acid position 705 is mutated to phenylalanine. Phosphorylation of Tyr705 is required for dimerization and nuclear translocation. When expressed at high levels, STAT3F has been shown to block the activation of endogenous STAT3 in various cell types, possibly by titrating out receptor docking sites (Fukada et al. 1996; Minami et al. 1996; Nakajima et al. 1996; Bonni et al. 1997; Ihara et al. 1997).

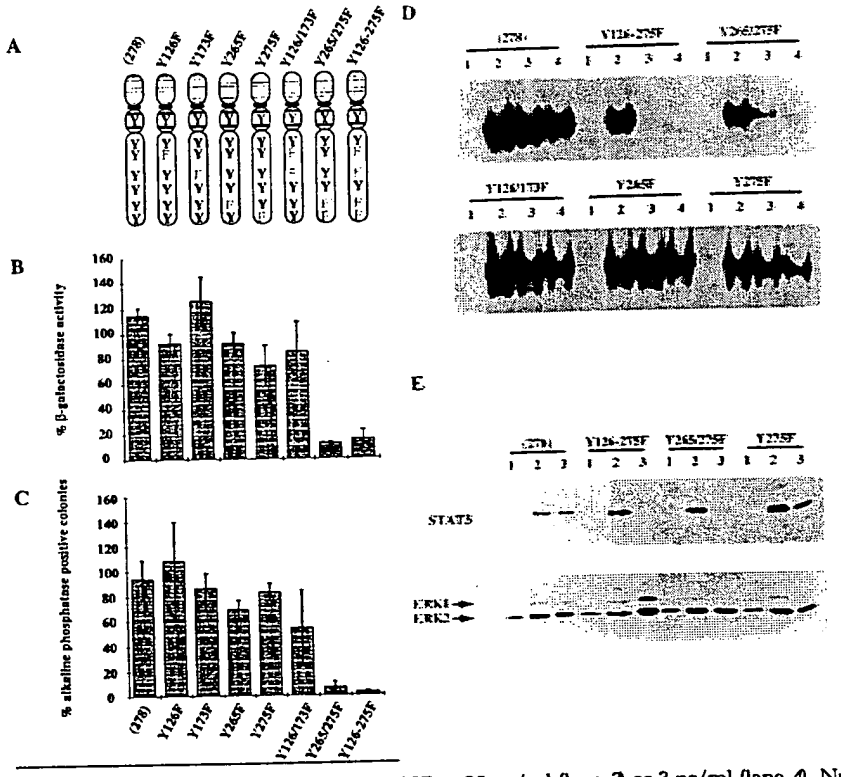

Figure 2. Effect of mutating STAT3 interaction sites in gp130 on ES cell self-renewal and induction of STAT3 DNA-binding activity. (*A*) Schematic of the various chimeric receptors indicating the tyrosine-phenylalanine substitutions introduced into the wild-type (278) gp130 cytoplasmic domain. Numbering commences with the first residue of the 278-amino-acid intracellular domain of mouse gp130. The phenylalanine (F) for tyrosine (Y) substitutions in the four STAT3 docking sites are indicated. The additional three tyrosines do not interact with STAT3 (Stahl et al. 1995). (*B*) Stem cell renewal mediated by chimeric receptors in response to G-CSF measured by β-galactosidase expression from the *Oct-4* locus. Data are mean ± S.E.M. for duplicate determinations on three independent clones normalized relative to response to IL-6/sIL-6R. (*C*) Efficiency of clonal stem cell renewal mediated by chimeric receptors in response to G-CSF measured by formation of alkaline phosphatase positive colonies. Data are mean ± S.E.M. for duplicate assays on three independent clones normalized relative to response to IL-6/sIL-6R. (*D*) Electrophoretic mobility-shift assay of induced STAT3 DNA binding. Transfected clones were left untreated (lane *1*) or stimulated for 30 min with IL-6/sIL-6R (lane *2*) or with G-CSF at 30 ng/ml (lane *3*) or 3 ng/ml (lane *4*). Nuclear extracts were assayed for SIE binding. (*E*) Immunoblot of STAT3 and ERK phosphorylation induced by G-CSF stimulation of chimeric receptors. Transfected clones were left untreated (lane *1*) or were stimulated for 20 min with IL-6/sIL-6R (lane *2*) or with G-CSF (lane *3*). Immunoblots of cell lysates were probed sequentially with antibodies specific for the active phosphorylated forms of ERK and STAT3.

Using conventional transfection approaches, we were unable to recover ES cell transfectants showing stable high-level expression of STAT3F. In parallel experiments, however, transfection of the LIF-independent embryonal carcinoma cell line P19 yielded multiple expressing clones. This suggested that blockade of STAT3 activation in ES cells specifically resulted in cell death, growth arrest, or differentiation. An alternative transfection and expression strategy was therefore adopted to enable characterization of the consequences of STAT3F expression. The approach, termed supertransfection, relies on expression of polyoma virus large T protein by the recipient ES cells and its interaction with a polyoma origin of replication present in the transfected DNA. This results in efficient episomal propagation of incoming plasmid (Gassmann et al. 1995). We have developed this system for efficent cDNA expression in ES cells (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.). The process yields at least 100-fold more stable transfectants than conventional transfection protocols. A second important advantage of episomal supertransfection is that the unpredictable effects of chromosomal integration are avoided, with the result that the level of expression is both stable and relatively uniform (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.).

The STAT3F mutant cDNA was introduced into the supertransfection vector pHPCAG. The wild-type STAT3 coding sequence was also introduced, in both sense and antisense orientations. The three constructs were electroporated into MG1.19 cells that harbor a large T expression plasmid and can be supertransfected with constructs containing the polyoma origin (Gassmann et al. 1995). Supertransfectants were isolated by selection in hygromycin B for 8 days in the presence of LIF. Colonies were fixed, stained with Leishman's reagent, counted, and scored for the presence of stem cells and differentiated cells. More than 95% of colonies obtained following supertransfection with control or wild-type STAT3 vector were stem cell colonies (Fig. 3A). A modest increase in the proportion of differentiated colonies was obtained with the antisense construct. The STAT3F vector, however, yielded predominantly differentiated colonies. A decrease in total number of colonies was also observed after supertransfection with STAT3F. This may reflect an early onset of differentiation that would produce very small clones that would not be scored. Alternatively, very high levels of STAT3F expression may also be toxic, though this has not been reported in other cell types. Morphologically, the differentiated STAT3F colonies closely resembled the differentiated colonies generated on culture of ES cells in the absence of LIF (Fig.

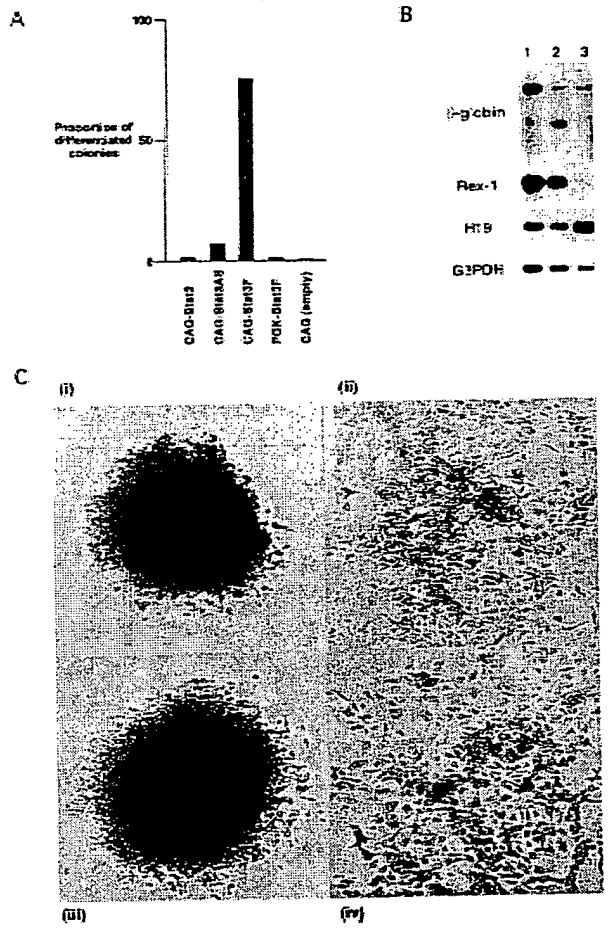

Figure 3. Induction of differentiation by expression of STAT3F in MG1.19 ES cells. (*A*) Proportion of differentiated colonies in LIF-supplemented medium resulting from supertransfection of STAT3, antisense STAT3, and STAT3F expression vectors. Colonies were fixed and stained with Leishman's reagent after 8 days of selection, and the numbers of stem cell colonies and differentiated colonies were scored. (*B*) Marker gene expression in STAT3F supertransfectants. Expression of marker genes in pools of MG1.19 cells supertransfected with STAT3 (lane *1*), STAT3 antisense (lane *2*), and STAT3F (lane *3*) expression vectors. Total RNA was prepared after 8 days of selection in LIF-supplemented medium, and 5-μg aliquots were analyzed by filter hybridization with β-globin, Rex-1, H19, and G3PDH probes. The β-globin probe detects all transgene mRNA species generated from pHPCAG, including an alternatively spliced product from the antisense contruct. (*C*) Photomicrographs of representative colonies 8 days after supertransfection with (*i*) STAT3, (*ii*) STAT3F, and (*iii*) empty expression vectors and selection in the presence of LIF, or (*iv*) induction of differentiation by culture in the absence of LIF for 8 days.

3C). Various other cDNAs have been expressed in ES cells using this system, with little or no effect on formation of stem cell colonies (data not shown). This suggested that the effect on differentiation was specifically attributable to expression of STAT3F.

The differentiation induced by expression of STAT3F was examined further by expression analysis of the marker genes *rex1* and *H19*. Rex-1 mRNA, which is specifically expressed in undifferentiated stem cells, was down-regulated in STAT3F supertransfectants. In contrast, H19 RNA, which is found at low levels in stem cells but is up-regulated during differentiation, was increased (Fig. 3B). A similar pattern of gene regulation is observed during differentiation of ES cells induced by withdrawal of LIF. These data confirm that the morphological differentiation triggered by STAT3F is accompanied by reprogramming of gene expression.

STAT3F was also expressed from the mouse phosphoglycerate kinase (*pgk-1*) promoter in the episomal vector pHPPGK. This vector gives at least 10-fold lower expression than pHPCAG (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.). In this case, there was no significant effect on either colony number or differentiation status of MG1.19 supertransfectants. A relatively high level of expression of the dominant interfering mutant therefore appears necessary to block self-renewal.

Effect of STAT3F on self-renewal is suppressed by coexpression of STAT3

To test whether the induction of differentiation by expression of STAT3F was due to an inhibition of endogenous STAT3 activity, we attempted to rescue the stem cell phenotype by coexpression of wild-type STAT3 and also of STAT1 and STAT4. A STAT3F expression vector carrying a blasticidin resistance marker was cosupertransfected into MG1.19 cells with episomal constructs for expression of wild-type STATs and hygromycin resistance. Cosupertransfectants were isolated in medium containing both 20 μg/ml blasticidin S and 80 μg/ml of hygromycin B. The numbers of stem cell and differentiated colonies were scored after 8 days. As shown in Figure 4, only coexpression of wild-type STAT3 restored self-renewal in the presence of STAT3F. Transfection with STAT1 or STAT4 constructs alone had no effect on self-renewal in the absence of STAT3F (not shown) and did not alter differentiation induced by STAT3F. In the case of supertransfection with the CAG promoter STAT1 construct, the total number of colonies (stem plus differentiated) recovered was reduced, but the relative proportion of stem cell colonies versus differentiated cells was unaltered. This occurred in both the presence and absence of coexpression of STAT3F and suggests that high-level expression of STAT1 may be toxic to ES cells. By using the mouse PGK-1 promoter to drive lower levels of expression (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.), comparable numbers of colonies were recovered on transfection with the STAT1 as with the other constructs. In this case, again only the STAT3 construct showed any restoration of stem cell colonies, although to a lower degree than with the high-expression CAG vector (not shown). These data indicate that STAT3 has a specific function in ES cells that cannot be compensated by STAT1 or STAT4 (see Discussion).

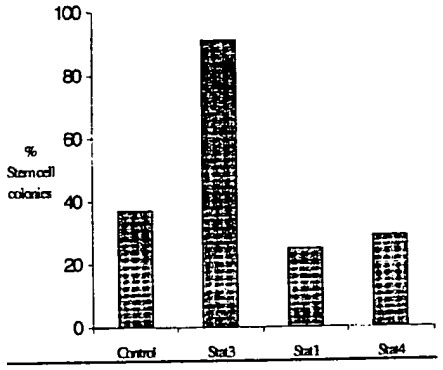

Figure 4. Cosupertransfection of STAT3F with wild-type STAT expression vectors. Proportions of undifferentiated stem cell colonies generated after cosupertransfection of MG1.19 ES cells with 10 μg of pBPCAGGS-STAT3F plus 10 μg of pHPCAG vector containing stuffer (control), STAT3, STAT1, or STAT4 inserts. After 8 days of selection with 80 μg/ml of hygromycin B plus 20 μg/ml of blasticidin S, colonies were fixed and stained with Leishman's reagent.

Generation of an inducible STAT3F transgene integration in ES cells

The effect of STAT3F expression on endogenous STAT3 activity could not be monitored directly in undifferentiated ES cells because ES cells expressing appreciable STAT3F constitutively could not be propagated. This required the generation of an inducible transgene. The tetracycline-regulatable system (tet-off) developed by Bujard and colleagues (Gossen and Bujard 1992) has been shown to confer inducibility on transgene expression in several cell types in culture and in the intact animal. However, it has proven problematic to establish this two-component system in ES cells. This is probably due to a combination of the relatively toxic effects of the tet repressor–VP16 fusion (tTA) and the tendency of ES cells to suppress expression of integrated transgenes (silencing). We have isolated previously an ES cell line, ZHTc6, that maintains stable production of effective but nontoxic levels of tTA from a gene trap integration (H. Niwa and A. Smith, in prep.). This cell line also contains a tetracycline-responsive hCMV*-1 transgene integrated at a favorable expression site. Expression of such transgenes is usually deregulated and/or mosaic in ES cells because of the sensitivity of the hCMV*-1 promoter to site of integration effects and silencing. However, transgene expression in line ZHTc6 is completely repressed in the presence of tetracycline but is activated in all cells on withdrawal of tetracycline as revealed by β-galactosidase reporter expression (H. Niwa and A. Smith, in prep.). Because of the low efficiency of establishing de novo transgene integrations with such favorable characteristics, we adopted a transgene substitution approach to generate an inducible STAT3F transgene.

A targeting vector was designed for introduction of the STAT3F sequences into the hCMV*-1 locus by homologous recombination, using 5′ and 3′ sequences from the original transgenic construct as homology arms (Fig. 5A). In the presence of tetracycline, ZHTc6 cells are sensitive to G418 because the hCMV*-1 promoter is repressed. Advantage was taken of this by including a constitutive MC1 enhancer/promoter in the supertargeting vector to drive selectable marker expression. The absence of the *neo* sequence, however, requires that a legitimate recombination event with the resident transgene occur to confer G418 resistance. This powerful selection facilitated the isolation of targeted clones in which the STAT3F sequence was faithfully integrated 3′ to the hCMV*-1 promoter (Fig. 5B). In the continued presence of tetracycline, the targeted cells were maintained readily as un-

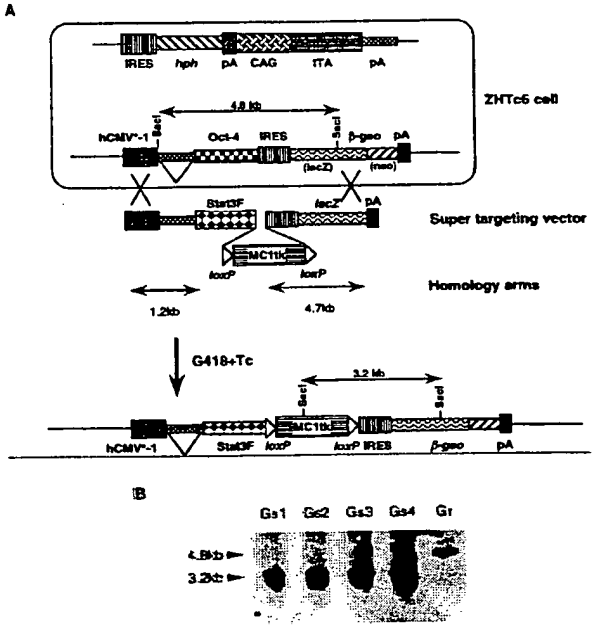

Figure 5. Generation of an inducible STAT3F transgene integration by supertargeting. (*A*) Schematic of supertargeting strategy for introduction of STAT3F into a tetracycline-regulatable expression site. ZHTc6 ES cells contain a tetracycline-regulated transgene comprising the hCMV*-1 promoter (Gossen and Bujard 1992), β-globin second intron, Oct-4 open reading frame (Okazawa et al. 1991), and IRESβgeopA selection marker (Mountford et al. 1994). Homologous recombination can be used to replace the Oct-4 sequence (supertargeting). Use of a truncated selection marker in the targeting vector facilitates the isolation of homologous recombinants. ZHTc6 cells were electroporated with the STAT3F-SuperKO vector and selected in G418 in the presence of tetracycline. G418-resistant clones were duplicated and screened for sensitivity against gancyclovir to enrich further for homologous recombinants. The option of excising the *loxP*-flanked MC1tk cassette by transient expression of Cre recombinase was not pursued. (*B*) Diagnosis of the supertargeting event in Gs ES cells. Gancyclovir-sensitive (Gs; lanes *1–4*) and -resistant (Gr; lane *5*) clones were analyzed by Southern hybridization. A 3.2-kb *Sac*I fragment was detected with a probe from the 5′ end of *lacZ* in the Gs samples, indicative of the correct replacement of the *Oct-4* cDNA sequence with STAT3F sequence. The Gr clone retained the 4.8-kb fragment diagnostic for the original *Oct-4* transgene integration in ZHTc6 cells.

differentiated stem cell colonies in the presence of LIF. Three clones, Gs1, Gs2, and Gs3, were then analyzed further.

Induced expression of STAT3F blocks ES cell self-renewal and causes differentiation

Withdrawal of tetracycline from Gs1, Gs2, or Gs3 cells resulted in the induction of differentiation in all three clones (Fig. 6A–C). Importantly, the efficiency of colony formation was not significantly different in the presence or absence of tetracycline, indicating that there is no toxic effect of STAT3F induction. The induced cultures differentiated over a 3- to 4-day time period, paralleling the behavior of parental ES cells on removal of LIF (Smith 1991). The differentiation response was confirmed by Northern hybridization analysis of Rex-1 and H19 transcripts (data not shown).

Mobility retardation analysis was used to investigate directly STAT3 activation in STAT3F-expressing ES cells. The data in Figure 6D show that the level of STAT3 DNA-binding activity induced by gp130 stimu-

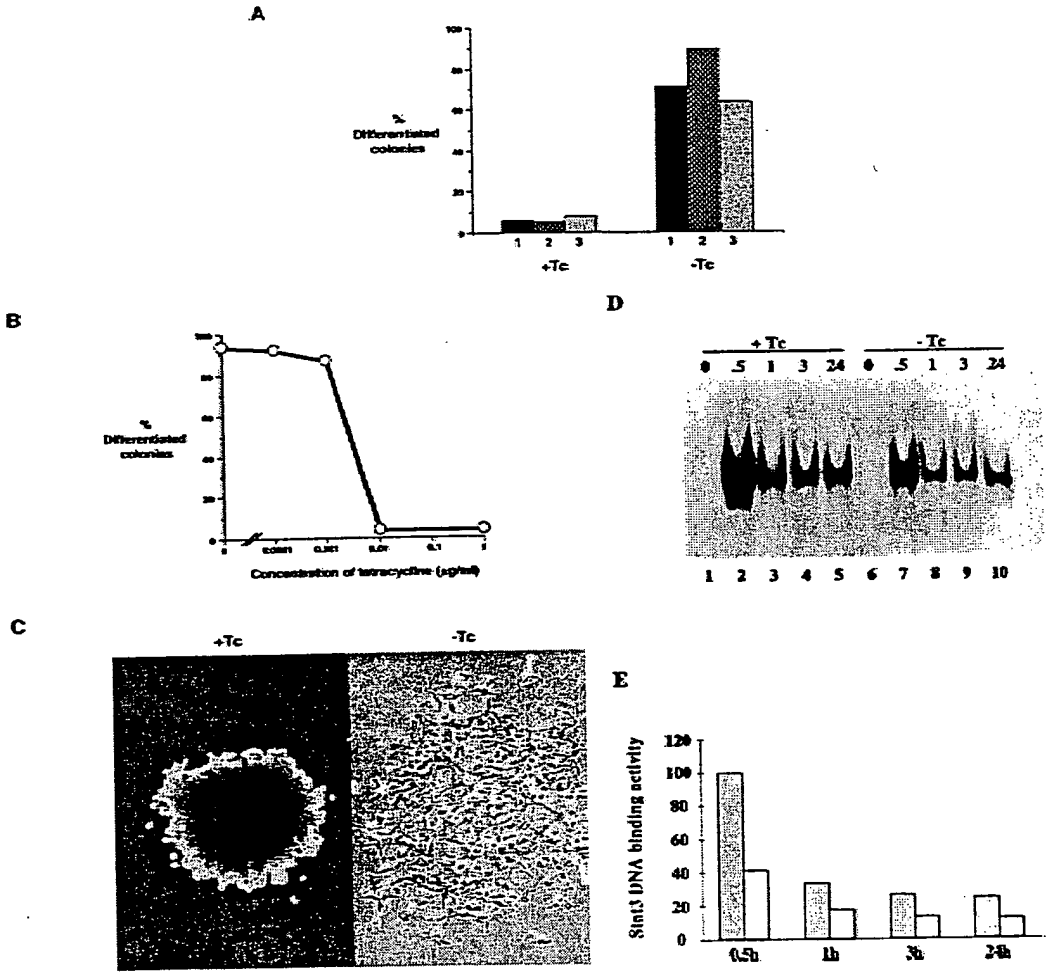

Figure 6. Induced expression of STAT3F causes ES cell differentiation and inhibits STAT3 activation. (*A*) Differentiation of Gs ES cells induced by withdrawal of tetracycline. Gs ES cells grown up in the presence of tetracycline were plated at clonal density (500 cells/60-mm dish) in LIF-supplemented medium in the presence or absence of tetracycline (1 μg/ml). After 6 days, colonies were fixed and stained with Leishman's reagent. The histogram records the proportions of differentiated colonies for three independent clones, Gs1 (solid bars), Gs2 (hatched bars), and Gs3 (shaded bars). (*B*) Dose response curve of Gs2 cell differentiation. Gs2 ES cells were cultured as above in the presence of the indicated concentrations of tetracycline, then fixed, stained, and scored. (*C*) Photomicrographs of uninduced and induced Gs2 ES cells. Representative colonies of Gs2 cells cultured for 6 days in LIF-supplemented medium in the presence (+Tc) or absence (–Tc) of tetracycline (1 μg/ml) and then fixed and stained with Leishman's reagent. (*D*) Mobility retardation assay of STAT3 DNA-binding activity in noninduced and induced Gs2 cells. Gs2 ES cells were cultured for 72 hr in the presence or absence of tetracycline. IL-6/sIL-6R was withdrawn for the final 24 hr, then restored for the indicated times. Nuclear extracts were prepared and assayed as described for SIE DNA-binding activity. (*E*) Quantitation of STAT3 SIE binding by PhosphorImager. (Shaded bars) +Tc; (open bars) –Tc.

lation was significantly lower in the presence of STAT3F. Quantitative PhosphorImager analysis confirmed a reduction of 50% or greater in the gel shift signal at all time points (Fig. 6E). The presence of residual STAT3 activity is consistent with the notion that a threshold level of active STAT3 is required to sustain self-renewal.

These findings confirm that expression of STAT3F in ES cells reduces gp130-mediated activation of STAT3, thereby blocking self-renewal and promoting differentiation.

Discussion

The primary cytoplasmic signal transduction event emanating from a ligand-activated LIF-R/gp130 complex in ES cells as in other cell types is considered to be transphosphorylation and activation of receptor-associated Janus kinases (JAKs) (Davis et al. 1993; Narazaki et al. 1994). The JAKs then phosphorylate tyrosine residues in the receptors, creating docking sites for SH2 domain-containing proteins, notably including the STAT factors STAT1 and STAT3 (Lutticken et al. 1994; Stahl et al. 1995). STAT proteins are themselves targets for phosphorylation by JAKs, which leads to their dimerization and translocation to the nucleus. Other signal transducing molecules can also be activated downstream of gp130, including insulin receptor substrate-1 (IRS-1), phosphoinositide-3 kinases (PI-3 kinase), nonreceptor tyrosine kinases such as Hck and Btk, the tyrosine phosphatase SHP2, and the mitogen-activated protein kinases ERK1 and ERK2 (Boulton et al. 1994; Ernst et al. 1994; Yin and Yang 1994; Argetsinger et al. 1995; Matsuda et al. 1995a,b). This modular signaling system has been assumed to underlie the diverse and pleiotropic effects of IL-6 and LIF-related cytokines in different cell types. A key issue therefore is to resolve the relative contribution of different signaling pathways in any given responsive cell type. A critical role has been ascribed to SHP2-mediated activation of the MAP kinase cascade in proliferation of BAF-BO3 cells (Fukada et al. 1996) and suppression of apoptosis in cardiomyocytes (Sheng et al. 1997). In contrast, the differentiation responses of myeloid M1 cells (Minami et al. 1996; Nakajima et al. 1996) and primary neural precursors (Bonni et al. 1997) are effected via activation of STAT3. Previous studies in ES cells have suggested that JAK–STAT signaling, ERK activation, and the nonreceptor tyrosine kinase Hck could all be involved in LIF signaling (Ernst et al. 1994, 1996; Narazaki et al. 1994; Hocke et al. 1995; Boeuf et al. 1997).

We initially investigated the ability of chimeric receptor constructs to signal ES cell self-renewal by isolation of stably expressing transfectants. The observation that G-CSF-R can support ES cell propagation drew attention to signaling features conserved between G-CSF-R and gp130, notably the induction of STAT3 DNA-binding activity. Combined substitutions of the tyrosine residues in the STAT3 binding sites of gp130 cytoplasmic domain were associated with different levels of STAT3 activation and indicated that a self-renewal signal is associated with a threshold of STAT3 activity. Moreover, the four STAT3 sites do not appear to act in either a redundant or simple cumulative manner. Both self-renewal signaling and induction of STAT3 DNA-binding activity were maintained on pairwise mutation of the two-membrane proximal STAT3 docking sites (Y126 and Y173) but not on mutation of the carboxy-terminal pair (Y265 and Y275) (see Fig. 2). This observation is somewhat unexpected as it has been shown previously that the isolated phosphopeptide sequences have equivalent STAT3 binding properties (Stahl et al. 1995) and that a truncated receptor with a single-membrane proximal STAT3 site (Y126) can efficiently induce STAT3-mediated differentiation of M1 cells (Yamanaka et al. 1996). It is important to note, however, that in the truncated receptor, sequences that mediate receptor internalization (Dittrich et al. 1996) have also been deleted with unpredictable consequences for signaling properties. Our findings indicate that in the normal context of the full-length receptor, the four STAT3 docking sites are not equivalent. The explanation for the reduced activity of the membrane proximal pair of sites is unclear though one possibility is that availability of Y126 may be influenced by interaction of SHP2 with Y118 (note enhanced ERK activation from Y126-275F chimera in Fig. 2E).

The finding that mutation of the STAT3 binding sites in the cytoplasmic domain of gp130 abolished the self-renewal signal prompted a direct investigation of the role of this transcription factor. New strategies were required to express the dominant interfering mutant STAT3F in ES cells. The methods we have deployed in this study enhance the experimental versatility and tractability of ES cells and establish new avenues for the characterization in vitro of gene functions involved in stem cell propagation, commitment, or differentiation. Because of the >100-fold increase in stable transfection efficiency and the relative homogeneity of expression (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.), episomal supertransfection provides a methodology for the screening and analysis of cDNAs whose expression is not compatible with ES cell self-renewal. The first demonstration of effective operation of the tetracycline regulation system in ES cells provides a complementary inducible expression approach. These two methods should find broad application in functional screening and in the genetic manipulation of lineage commitment and differentiation processes in ES cells.

Both constitutive expression of STAT3F following episomal supertransfection and induced expression from the regulatable chromosomal site inhibited self-renewal and resulted in differentiation. The episomal approach also allowed the specificity of the requirement for STAT3 to be established by coexpression of various STAT family members with STAT3F. The finding that STAT3 can restore self-renewal indicates that this factor serves a specific and nonredundant function in ES cell self-renewal in response to LIF. The evidence that STAT1 cannot compensate for STAT3 is noteworthy because STAT1 can be activated in response to LIF in ES cells, though to a much lesser extent than STAT3 (Starr

et al. 1997). STAT1 may play little or no role in ES cell propagation. Induction of STAT1 DNA-binding activity was not evidently associated with self-renewal signaling from the various chimeric receptors used in this study (Figs. 1B and 2D). Furthermore, ES cells in which both alleles of the *stat1* gene have been inactivated are phenotypically normal (Durbin et al. 1996).

A role for STAT3 in ES cell signaling has recently also been suggested by Boeuf et al. (1997) who reported the isolation of ES cell clones expressing STAT3F constitutively. These cells apparently showed an increased tendency to differentiate after 1 month or more in culture. The basis of this phenomenon is unclear because absence or blockade of LIF signaling results in complete differentiation within a few days (Smith et al. 1988; Williams et al. 1988; C. Dani, I. Chambers, S. Johnstone, M. Robertson, B. Ebrahimi-Chahardahcherik, M. Saito, T. Taga, M. Li, T. Burdon, J. Nichols, and A.G. Smith, in prep.). We were unable to establish conventional transfectants expressing significant levels of STAT3F. However, our data on both episomal and induced expression demonstrate that STAT3F rapidly and efficiently blocks ES cell self-renewal and triggers differentiation.

Our results establish that STAT3 activation is essential for LIF-R/gp130-mediated ES cell self-renewal. STAT3 activity is regulated by phosphorylation on both tyrosine and serine (Wen et al. 1995), and a constitutively active mutant has not been described. An isoform of STAT3, STAT3β, generated by alternative splicing, is reported to show sustained activation properties (Schaefer et al. 1995). ES cells supertransfected with a STAT3β vector remained LIF dependent (data not shown), however, indicating that this isoform does not substitute for activated STAT3 in ES cells. This may be because STAT3β appears to function by formation of heterodimers with c-Jun (Schaefer et al. 1995), and it is anticipated that the STAT3β/c-Jun complex regulates a distinct spectrum of target genes compared with the STAT3 homodimer. It is noteworthy, however, that expression of v-*src* in ES cells renders them LIF independent (Boulter et al. 1991). v-Src has been shown to associate with and cause constitutive activation of STAT3 (Cao et al. 1996).

The p42/p44 MAP kinase pathway (ERK1 and ERK2) has been reported to be activated by LIF in ES cells as in other cell types (Ernst et al. 1996; Boeuf et al. 1997). The Ras–ERK cascade is coupled to gp130 via the adaptor molecule SHP2 (Fukada et al. 1996; Yamanaka et al. 1996). SHP2 interacts with activated gp130 at phosphorylated tyrosine residue 118 (Stahl et al. 1995). Significantly, mutation of this residue does not inhibit self-renewal signaling in ES cells (T. Burdon, I. Chambers, C. Stracey, J. Nichols, and A.G. Smith, in prep.). Furthermore, the MEK inhibitor PD098059 (Dudley et al. 1995) that specifically blocks activation of the ERK kinases does not inhibit stem cell colony formation in response to LIF (T. Burdon, I. Chambers, C. Stracey, J. Nichols, and A.G. Smith, in prep.). Thus, although contributions of other pathways are not precluded, STAT3 appears to play a central role in ES cell self-renewal. The underlying importance of STAT3 is further attested to by the finding that homozygous disruption of the *Stat3* gene in mice is associated with early embryonic lethality (Takeda et al. 1997).

It is striking that the role of STAT3 in propagation of the undifferentiated pluripotential phenotype of ES cells contrasts with previously characterized functions as an effector of somatic cell differentiation. Dominant interfering mutants of STAT3 have been shown to block macrophage differentiation of myeloid M1 cells induced by IL-6 or LIF (Minami et al. 1996; Nakajima et al. 1996) or by GCSF (Shimozaki et al. 1997). STAT3 activation has similarly been shown to mediate IL-6- or LIF-induced astrocytic differentiation of primary cortical neuroepithelial cells (Bonni et al. 1997). Recently it has also been shown that STAT3 is activated by hepatocyte growth factor and mediates epithelial tubulogenesis (Boccaccio et al. 1998). STAT3 thus has distinct effects in different cell types. A common theme, however, may be the regulation of genes that determine cell identity. The diverse effects of the LIF/IL-6 family of cytokines on cellular differentiation and gene expression appear to reflect cell-type specific effects of active STAT3. In the context of stem cell propagation, the key issue now is to identify transcriptional targets of STAT3 in ES cells and to illuminate the relationship between STAT3 and the essential ES cell-specific transcription factor Oct-4.

Materials and methods

Cell culture and transfection

ES cells were maintained in the absence of feeder cells in Glasgow modification of Eagle medium (GMEM) supplemented with fetal calf serum, 2-mercaptoethanol, and LIF (Smith 1991). CGR8 (Mountford et al. 1994) and MG1.19 (Gassmann et al. 1995) ES cells have been described elsewhere. DO27 ES cells have had both copies of the *lif* gene inactivated by homologous recombination and the IRESβgeo selection marker/reporter inserted into the *oct4* gene as described (C. Dani, I. Chambers, S. Johnstone, M. Robertson, B. Ebrahimi-Chahardahcherik, M. Saito, T. Taga, M. Li, T. Burdon, J. Nichols, and A.G. Smith, in prep.). LRKOh34 ES cells have targeted disruptions in both copies of the *lifr* gene (M. Li, I. Chambers, J. Nichols, and A.G. Smith, in prep.) and are maintained in medium in which LIF is substituted with IL-6 (50 ng/ml) and soluble IL-6 receptor (5% CHO-5E7 conditioned medium; Yasukawa et al. 1990). For conventional transfection with pPCAGIZ vectors, 1×10^7 cells were electroporated with 100 μg of linearized plasmid DNA at 800 V and 3 μF in a 0.4-cm cuvette using a Bio-Rad gene pulser and then selected in the presence of zeocin (Invitrogen). For transfection of episomal vectors (supertransfection), 5×10^6 MG1.19 cells were electroporated with 20 μg of supercoiled plasmid DNA at 200 V and 960 μF and then cultured in the presence of either 80 μg/ml hygromycin B (Boehringer Mannheim) or 4–20 μg/ml blasticidin S (Waken Seiyaku), or both hygromycin plus blasticidin for cosupertransfection.

Generation of tetracycline regulatable transgenes in ES cells

ZHTc6 ES cells were derived from CGR8 ES cells (Mountford et al. 1994) and will be described in detail elsewhere (H. Niwa and A.G. Smith, in prep.). They carry a targeted integration of IRES*zeo* in one *Oct3/4* allele. They also carry a gene trap inte-

gration of an IRES*hph*:CAGtTA construct that confers stable expression of the tetracycline-responsive tTA transactivator and a randomly integrated hCMV*-1–Oct4–IRESβgeopA transgene. These cells were routinely maintained in the presence of 10 μg/ml zeocin and 1 μg/ml tetracycline-HCl (Sigma).

The hCMV*-1–Oct-4-IRESβgeopA transgene is comprised of the tetracycline-inducible promoter hCMV*-1 derived from pUHD10-3 (Gossen and Bujard 1992), rabbit β-globin second intron, full-length Oct-4 cDNA, and IRESβgeopA unit (Mountford et al. 1994). pSuperKO (see Fig. 5A) contains the hCMV*-1 and rabbit globin sequences as the 5′ homology arm and the IRES*lacZ* cassette as 3′ arm. Intervening are a stuffer sequence with *Xho*I and *Sfi*I cloning sites and a *loxP*-flanked MC1*tk* cassette (Mansour et al. 1988). The STAT3F cDNA was introduced as a *Sal*I fragment between the *Xho*I sites. For gene targeting, 2×10^7 cells were electroporated with 100 μg linearized SuperKO-STAT3F plasmid DNA at 800 V and 3 μF and then selected in the presence of 200 μg/ml G418 (GIBCO BRL) and 1 μg/ml tetracycline-HCl. Targeted clones were maintained in the continuous presence of tetracycline-HCl.

Plasmid construction

DNA manipulations were performed by standard procedures (Sambrook et al. 1989). Full details of plasmid constructions are available on request. The full-length mouse G-CSF-R cDNA (pJ17) was provided by Shigekazu Nagata (Fukunaga et al. 1990), and the G-CSF-R/LIF-R chimeric receptor construct (Baumann et al. 1994b) was provided by Steve Ziegler. G-CSF-R/gp130 chimeric receptor constructs were generated by fusing the coding sequence for the extracellular domain of human G-CSF-R (Baumann et al. 1994b) to an *Eco*RI fragment encoding the transmembrane domain and the entire cytoplasmic region of mouse gp130 cDNA (Hibi et al. 1990). Phenylalanine substitutions were introduced into the intracellular domain of gp130 by PCR overlap mutagenesis (Higuchi et al. 1988). PCR products were substituted into the G-CSF-R/gp130 chimera and sequenced. Episomal expression vectors pHPCAG, pBPCAG, and pHPPGK are described elsewhere (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.). The expression vector pPCAGIZ, which can be used as both an episomal and an integrated expression vector, was constructed by ligation of the encephalomyocarditis virus IRES (pCITE-1, Novagen) with the *Streptoalloteichus* bleomycin resistant gene (*Sh ble:zeo*) from pZeoSV (Invitrogen) and introduction into pPCAG (H. Niwa, I. Chambers, L. Forrester, M. Gassmann, and A.G. Smith, in prep.). cDNAs are inserted into a *Xho*I site 5′ to the IRES. The requirement for continuous relatively high-level expression of the *zeo* gene to confer antibiotic resistance allows direct selection for integrations into favorable expression sites. Consequently, using this vector, ES cell transfectants can readily be isolated that sustain stable transgene expression (H. Niwa, T. Burdon, I. Chambers, and A.G. Smith, unpubl.).

RNA and DNA hybridization analyses

Total RNA (Chomczynski and Sacchi 1987) was separated on a 0.66 M formaldehyde, 0.8% agarose gel and blotted onto nylon membranes (Hybond N, Amersham). Hybridization was performed with β-globin third exon, Rex-1, H19, and GAPDH cDNA probes labeled by random hexamer primed DNA synthesis in the presence of [α-^{32}P]dCTP (3000 Ci/mmole).

For identification of targeted ES cell clones, genomic DNA was digested with *Sac*I, separated on a 0.7% agarose gel, and analyzed by nonradioactive filter hybridization (Gene Image, Amersham) with an *Eco*RI–*Sac*I fragment of the *lacZ* gene.

G-CSF-R binding assay

ES cells (1×10^6) were seeded in wells of a 24-well plate and grown for 24 hr. The cells were then cooled to 4°C and growth medium was replaced with 0.25 ml of ice-cold binding buffer (GMEM, 25 mM HEPES at pH 7.2, 0.2% BSA) containing 0.212 nM ^{125}I-labeled G-CSF-R (Amersham) in the presence or absence of a 1000-fold molar excess of cold G-CSF-R. Binding reactions were incubated for 3 hr at 4°C and terminated by washing the cells three times with ice-cold binding buffer. Cells were then solubilized in 0.5% NP-40, and an aliquot was counted in a gamma counter. All treatments were performed in duplicate. No specific binding was detected to untransfected cells, and nondisplaceable binding was consistent between clones.

Self-renewal assays

To measure self-renewal of ES cells at cloning density, cells were plated at 1000 cells per well (~100 cells/cm^2) in 6-well dishes and cultured for 6 days. Cells were either grown in the absence of cytokines, in 100 U/ml recombinant LIF (Smith 1991), in 100 ng/ml IL-6 plus soluble IL-6R, or in 30 ng/ml G-CSF-R, as appropriate. On day 6, colonies were fixed and stained with Leishman's reagent (Smith 1991) or for alkaline phosphatase activity (Sigma leukocyte alkaline phosphatase kit) (Bernstine et al. 1973). Numbers of stem cell and differentiated colonies were scored by microscopic examination, in some cases with computer-assisted image analysis. All assays were performed in duplicate or triplicate.

Stem cell-specific expression of β-galactosidase from the *oct4* locus in D027 cells was quantified by ONPG assay on triplicate samples. Cells were plated at 5000 per well in 24-well dishes and cultured for 6 days in the presence or absence of cytokine as above. On day 6, cells were washed once with PBS and lysed in 0.4 ml of 0.25 M Tris (pH 7.5), 5 mM DTT, and 0.5% NP-40. Lysate (40 μl) was mixed with 100 μl of ONPG buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 1.2 mM ONPG) in a microtiter plate and incubated at 37°C for 2–4 hr, and the absorbance was read at 420 nm.

Preparation of nuclear extracts and band-shift assays

One day after plating (1×10^6 cells per 60-mm dish), ES cells were washed with PBS and refed with medium lacking cytokines. The next day, cells were stimulated with IL-6 (100 ng/ml plus soluble receptor) or G-CSF-R (30 ng/ml) for 30 min, washed with ice-cold PBS, scraped off the plates, and collected by centrifugation. Nuclear extracts were prepared by the method described (Gobert et al. 1996) except that protease inhibitors (aprotinin, pepstatin, and leupeptin) were omitted from the cell lysis buffer. Protein concentrations of nuclear extracts were determined using a Bradford assay (Bio-Rad). Aliquots (2 μg) of nuclear extract were incubated with 0.25 ng of ^{32}P-labeled double-stranded SIEm67 oligonucleotide probe (Sadowski et al. 1993) in binding buffer (20 mM HEPES at pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% NP-40, 10% glycerol, 2 μg/ml of poly[d(I-C)], and 1 mg/ml BSA) for 20 min at room temperature. Binding reactions were resolved by electrophoresis on a prerun 5% polyacrylamide gel in 0.25× TBE for 3 hr. Gels were fixed in 10% acetic acid, dried under vacuum, and subjected to autoradiography or quantitated on a Bio-Rad PhosphorImager.

Immunoblotting

One day after plating (1×10^6 cells per 60-mm dish), ES cells were refed with medium containing 1% FCS and lacking cyto-

kines. Following overnight incubation, cells were transferred to serum-free medium for 4 hr prior to stimulation with IL-6 (100 ng/ml plus soluble receptor) or G-CSF-R (30 ng/ml) for 20 min. Cells were then washed once with ice-cold PBS and lysed on ice in 100 µl SDS sample buffer. Ten- microliter aliquots of the lysates were fractionated on a 10% SDS–polyacrylamide gel and electroblotted onto nitrocellulose. After overnight treatment in blocking buffer (25 mM Tris-HCl at pH 7.4, 2.7 mM KCl, 140 mM NaCl, 0.1% Tween 20, 5% nonfat dried milk), membranes were probed sequentially with the phospho-specific anti-ERK and anti-STAT3 antibodies according to the directions provided by the supplier (New England Biolabs). Blots were incubated with HRP-coupled anti-rabbit IgG and developed using ECL reagents (Amersham). Membranes were stripped between probings by incubation at 50°C for 30 min in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol.

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Differences between human and mouse embryonic stem cells

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Abstract

We compared gene expression profiles of mouse and human ES cells by immunocytochemistry, RT-PCR, and membrane-based focused cDNA array analysis. Several markers that in concert could distinguish undifferentiated ES cells from their differentiated progeny were identified. These included known markers such as SSEA antigens, OCT3/4, SOX-2, REX-1 and TERT, as well as additional markers such as UTF-1, TRF1, TRF2, connexin43, and connexin45, FGFR-4, ABCG-2, and Glut-1. A set of negative markers that confirm the absence of differentiation was also developed. These include genes characteristic of trophoectoderm, markers of germ layers, and of more specialized progenitor cells. While the expression of many of the markers was similar in mouse and human cells, significant differences were found in the expression of vimentin, β-III tubulin, alpha-fetoprotein, eomesodermin, HEB, ARNT, and FoxD3 as well as in the expression of the LIF receptor complex LIFR/IL6ST (gp130). Profound differences in cell cycle regulation, control of apoptosis, and cytokine expression were uncovered using focused microarrays. The profile of gene expression observed in H1 cells was similar to that of two other human ES cell lines tested (line I-6 and clonal line-H9.2) and to feeder-free subclones of H1, H7, and H9, indicating that the observed differences between human and mouse ES cells were species-specific rather than arising from differences in culture conditions.

Keywords: Embryonic stem cells; Human; Mouse; Markers; RT-PCR; cDNA microarray; Leukemia inhibitory factor; Apoptosis; Cell cycle; Cytokines

Introduction

Embryonic stem cell (ES cell) lines were first generated from mouse blastocysts by culturing inner cell mass (ICM) from pre-implantation embryos on feeder layers (Evans and Kaufman, 1981; Martin, 1981). The resulting cultures contained populations of cells, which grew as colonies, showed extensive capacity for replication, and were pluripotent as demonstrated by their ability to generate chimeras and transgenic mice and to differentiate in culture into ectodermal, endodermal, and mesodermal derivatives. Much has been learned from the generation of mouse ES cell lines in terms of methods of propagation, growth factor dependence, and marker expression. Mouse ES cells express genes characteristic of the early blastocyst such as the POU domain transcription factor OCT3/4 (Rosner et al., 1990; Scholer et al., 1990), the homeobox domain transcription factor SOX-2 (Yuan et al., 1995), the zinc finger protein REX-1 (Rogers et al., 1991), the transcriptional activator UTF-1 (Okuda et al., 1998), as well as carbohydrate epitopes SSEA-1, and SSEA-3 identified using specific antibodies at the preimplantation embryo stage (Krupnick et al., 1994). Mouse ES cells do not differentiate into trophoectoderm (Edwards, 2002), they can be propagated in continuous culture on a feeder layer of mouse embryonic fibroblasts or without feeders in the presence of leukemia inhibitory factor (LIF) (Smith and

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Hooper, 1987; Smith et al., 1988; Williams et al., 1988) or LIF-related cytokines (Conover et al., 1993; Pennica et al., 1995; Rose et al., 1994; Wolf et al., 1994), retain high telomerase levels, show karyotypic stability, and retain the ability to contribute to chimeras and form teratomas (reviewed in Burdon et al., 2002; Edwards, 2002; Gardner, 2002; Prelle et al., 2002; Rossant, 2001) after multiple passages in culture.

More recently, primate and human ES cell lines have been isolated. The first success was reported by Thomson et al. who isolated several monkey (Thomson and Marshall, 1998; Thomson et al., 1998b) and subsequently five human ES cell lines (Thomson et al., 1998a). This result has been replicated by several laboratories (reviewed in Carpenter et al., 2003), and sixty-four different derivations are now listed in the NIH registry for research use (http://escr.nih.gov). Currently, fewer than 10 cell lines are available in sufficient numbers for analysis, and only limited data on the fundamental properties of these lines are available. Recent studies, while limited, suggest that differences exist between mouse and human ES cell lines. Similar to mouse ES cells, which express mouse-specific embryonic antigens such as SSEA-1, at least some of the human ES cell lines express surface markers (glycolipids and glycoproteins) that were originally identified on human embryonic carcinoma cells (EC cells), such as SSEA-3, SSEA-4, TRA-1-81, TRA-1-60 (Draper et al., 2002; Henderson et al., 2002). On the other hand, human ES cells, unlike their mouse counterparts, do not appear to require LIF for their propagation or for maintenance of pluripotency (Reubinoff et al., 2000; Thomson et al., 1998a). Whether LIF has different effects or its action is actively inhibited in human ES cells as has been proposed in EC cells (Schuringa et al., 2002) remains to be determined. Furthermore, in contrast to mouse ES cells (Niwa et al., 2000; Rossant, 2001), human ES cells are able to differentiate into trophoblast-like cells (Odorico et al., 2001; Thomson et al., 1998a). Telomerase biology is different between mouse and humans (Forsyth et al., 2002) and differences in telomerase regulation in ES cells are likely, though no reliable comparisons exist. These differences suggest that caution must be exercised in extrapolation of the data that has accumulated on the properties of mouse ES cells to studies using human ES cells, and further studies analyzing the similarities and differences between mouse and human lines are required.

In this manuscript, we have compared a commonly used mouse ES cell line (D3) with the most easily available human ES cell line (H1) to assess similarities and differences when the cells are grown under similar conditions by the same laboratory. We show that several of the markers used to assess mouse ES cells can be used to examine human ES cells as well. Differences exist, however, in morphology, patterns of embryonic antigen immunostaining, expression of differentiation markers, as well as expression profiles of cytokines, cell cycle, and cell death-regulating genes. These differences were consistent across three different human ES cell lines tested and suggest that fundamental species-specific differences exist.

Methods

Cell culture

Human ES cell line H1 was obtained from WiCell Research Institute, Inc., (Madison, WI) and cultured according to their instructions. Briefly, cells were cultured on mouse embryonic fibroblast (MEF) feeders in ES cell medium consisting of DMEM/F12 (Invitrogen/GIBCO 11330-032) supplemented with 20% knockout serum replacement, 100 mM MEM nonessential amino acids, 0.55 mM beta-mercaptoethanol, 2 mM L-glutamine, antibiotics/antimycotics, and with 4 ng/ml human basic fibroblast growth factor (bFGF) (all from Invitrogen/GIBCO). MEF's derived from E12.5 mouse embryo were purchased from StemCell Technologies, Inc. (Vancouver, Canada). MEF were expanded on 0.5% bovine gelatin-coated dishes in DMEM medium (Invitrogen/GIBCO, cat# 11965-092) supplemented with 10% FBS (heat inactivated, Hyclone cat# 3007103), 2 mM glutamine and 100 mM MEM nonessential amino acids. Subconfluent MEF's cultures were treated with 10 μg/ml Mitomycin C (Sigma, St. Louis, MO) for 3 h to arrest cell division, trypsinized, and plated at $2 \times 10^4/cm^2$ onto 0.5% bovine gelatin-coated dishes overnight. Feeders were washed twice with PBS, and then incubated in ES cell medium for at least 1 h before plating ES cells. MEF's of passages 2–3 were used as feeders. ES cells plated on top of MEF feeders were cultured at 37°C in the atmosphere of 5% CO_2/5% O_2 within a humidified tissue culture incubator from Thermo Forma. H9.2 and I-6 cells were cultured at 21% O_2. When confluent (8–10 days after plating), ES cells were treated with 1 mg/ml collagenase, type IV (Invitrogen/GIBCO) for 5–10 min and gently scrapped off with 5-ml pipette. Cells were spun at 500 rpm for 3 min and the pellet was replated at 1:2–3 split or used for RNA purification.

Mouse ES cell line D3 was obtained from ATCC and were first expanded on fibroblast feeders treated with 10 μg/ml Mitomycin C as described above. In some experiments, STO-1 feeder cells (also from ATCC) were used. Subconfluent D3 cultures were trypsinized and replated (1:3 split) onto 0.5% bovine gelatin-coated tissue culture plates in the presence of 1,400 U/ml of leukemia inhibitory factor (LIF) (Chemicon, ESG1106) in mouse ES cell medium consisting of knockout Dulbecco's minimal essential medium (DMEM) (Invitrogen/GIBCO 10829-018) supplemented with 15% ES-qualified FBS (Invitrogen/Gibco 16141-061), 100 mM MEM nonessential amino acids, 0.55 mM beta-mercaptoethanol, 2 mM L-glutamine, and antibiotics (all from Invitrogen/GIBCO). When confluent (2–3 days after plating), D3 cells were harvested by trypsinization and replated for immunostaining

Table 1
Primers used for RT-PCR

GENE	UniGene	PRIMERS	References	UniGene	PRIMERS	References
ABCG-2	Mm.196728	F: CCATAGCCACAGGCCAAAGT R: GGGCCACATGATTCTTCCAC	This laboratory	Hs.194720	F: GTTTATCCGTGGTGTGTCTGG R: CTGAGCTATAGAGGCCTGGG	(Zhou et al., 2001)
Cx 43	Mm.4504	F: TACCACGCCACCACTGGCCCA R: ATTCTGGTTGTCGTCGGGGAAATC	This laboratory	Hs.74471	F: TACCATGCGACCAGTGGTGCGCT R: GAATTCTGGTTATCATCGGGGAA	(Chang et al., 1999)
Cx 45	Mm.3096	F: GGGCAAACCAATTCCACCACC R: CAAGATTAAATCCAGACGGAG	This laboratory	Hs.283746	F:CTATGCAATGCGCTGGAAACAACA R: CCCTGATTTGCTACTGGCAGT	(Chang et al., 1999)
FGFR-4	Mm.4912	F: TCCGACAAGGATTTGGCAG R: GCACTTCCGAGACTCCAGATAC	This laboratory	Hs.165950	F: GTTTCCCCTATGTGCAAGTCC R: GCGCTGCTGCGGTCCATGT	(Tartaglia et al., 2001)
FOXD3	Mm.4758	F: TCTTACATCGCGCTCATCAC R: TCTTGACGAAGCAGTCGTTG	(Hanna et al., 2002)	Hs.120204	F: CGACGACGGGCTGGAGGAGAA R: ATGAGCGCGATGTACGAGTA	This laboratory
Glut-1	Rn.30044	F: CAGTCAGCAATGAAGTCCAG R: AGCAGTAAGTTCTCAGCCTC	This laboratory	Hs.169902	F: GTGTTCGGCCTGGACTCCAT R: AGTGGGAAGAAGGCCAGGGCT	(Baroni et al., 1998)
OCT3/4	Mm.17031	F: GGAGAGGTGAAACCGTCCCTAGG R: AGAGGAGGTTCCCTCTGAGTTGC	(Anderson et al., 1999)	Hs.2860	F: CTTGCTGCAGAAGTGGGTGGAGGAA R: CTGCAGTGTGGGTTTCGGGCA	This laboratory
REX-1	Mm.3396	F: GGCCAGTCCAGAATACCAGA R: GAACTCGCTTCCAGAACCTG	(Thompson and Gudas, 2002)	Hs.169832	F: TGAAAGCCCACATCCTAACG R CAAGCTATCCTCCTGCTTTGG	This laboratory
SOX2	Mm.4541	F: GTGGAAACTTTTGTCCGAGAC R: TGGAGTGGGAGGAAGAGGTAAC	This laboratory	Hs.816	F: ATGCACCGCTACGACGTGA R CTTTTGCACCCCTCCCATTT	This laboratory
TERF1	Mm.4306	F: TTCAACAACCGAACAAGTGTC R: TCTCTTTCTCTTCCCCCTCC	(Klapper et al., 2001)	Hs.194562	F: GCAACAGCGCAGAGGCTATTATT R: AGGGCTGATTCCAAGGGTGTAA	(Yajima et al., 2001)
TERF2	Mm.6402	F: GCCCAAAGCATCCAAAGAC R: ACTCCATCCTTCACCCACTC	(Klapper et al., 2001)	Hs.100030	F: AAACGAAAGTTCAGCCCCG R: TCCTCCAAGACCAATCTGCTTA	(Yajima et al., 2001)
TERT	Mm.10109	F: CTGCGTGTGCGTGCTCTGGAC R: CACCTCAGCAAACAGCTTGTTCTC	(Klapper et al., 2001)	Hs.115256	F: AGCTATGCCCGGACCTCCAT R: GCCTGCAGCAGGAGGATCTT	This laboratory
AFP	Mm.80	F: GTTTTCTGAGGGATGAAACCTATG R: GAAGCTCTTGTTTCATGGTCTGTA	(Cantz et al., 2003)	Hs.155421	F: AAATACATCCAGGAGAGCCA R: CTGAGCTTGGCACAGATCCT	(Lafuste et al., 2002)
Actc1	Mm.195067	F: ACAATGTCCTATCTGGAG R: GTACAATGACTGATGAGAGA	This laboratory	Hs.119000	F: CACTGAATCCGCCTACCTCC R: TCGTGCCTCTACACCAGACC	(Karkkainen et al., 2002)
Brachyury	Mm.913	F: GCTGTGACTGCCTACCAGCAGAATG R: GAGAGAGAGCGAGCCTCCAAAC	This laboratory	Hs.143507	F: TAAGGTGGATCTTCAGGTAGC R: CATCTCATTGGTGAGCTCCCT	(Gokhale et al., 2000)
brain1	Rn.11354	F: CACAGCCCGCCCTCCT R: CAGAACCAGACCCGCACGAC	This laboratory	Hs.210862	F: TGGACTCAACAGCCACGAC R: TGAACGCTCGTCTGCAGC	This laboratory
HNF3-b	Mm.938	F: GGACGTAAAGGAAGGGACTCCAC R: GCAGCCCATTTGAATAATCAGCTCAC	(Maeyama et al., 2001)	Hs.155651	F: GACAAGTGAGAGAGCAAGTG R: ACAGTAGTGGAAACCGGAG	This laboratory
Krt1-14	Mm.6974	F: ATTCTCCTCATCCTCTCAAT R: GACAAGGGTCAAGTAAAGAG	This laboratory	Hs.455013	F: AGCTCCATGAAGGGCTCCTG R: CATGGTCACCTTCTCACTGC	(Werner and Munz, 2000)
Krt1-15	Mm.38498	F: CACCACATTCTTGCAAAC R: ATTAAGGTTCTGCATGGTC	This laboratory	Hs.418220	F: GGAGGTGGAAGCCGAAGTAT R: GAGAGGAGACCACCATCGCC	(Werner and Munz, 2000)
Msx1	Mm.259122	F: GCTATGACTTCTTTGCCACTCG R: TTAAGAGAAGGGGACCAGGTGG	(Wang and Sassoon, 1995)	Hs.424414	F: CCTTCCCTTTAACCCTCACAC R: CCGATTTCTCTGCGCTTTTC	This laboratory
Myf5	Mm.4984	F: TGCCATCCGCTACATTGAGAG R: CCGGGGTAGCAGGCTGTGAGTTG	(Kruger et al., 2001)	Hs.178023	F: GCCCGAATGTAACAGTCCTGTC R: TAAGCCTGGAACTAGAAGCCCC	This laboratory

Nestin	Mm.23742	F: AGTGTGAAGGCAAAGATAGC R: TCTGTCAGGATTGGGATGGG	This laboratory	X65964	F: CAGCGTTGGAACAGAGGTTGG R:TGGCACAGGTGTCTCAAGGGTAG	(Ignatova et al., 2002)
beta 3 tubulin	Mm.40068	F: TCACTGTGCCTGAACTTACC R: GGAACATAGCCGTAAACTGC	This laboratory	Hs.159154	F: CAACAGCACGGCCATCCAGG R: CTTGGGGCCCTGGGCCTCCGA	(Ranganathan et al., 1996)
vimentin	Mm.7	F: AAGGGTGAGTAGAGAGTTC R: AACACTGTTAGGAAAGAGG	This laboratory	Hs.297753	F:GACACTATTGGCCGCCTGCGGATGAG R: CTGCAGAAAGGCACTTGAAAGC	(Nishio et al., 2001)
Pdx1	Mm.4949	F: CGGACATCTCCCCATACG R: AAAGGGAGCTGGACGCGG	(Laybutt et al., 2002)	Hs.32938	F: GTCCTGGAGGAGCCCAAC R: GCAGTCCTGCTCAGGCTC	(Itkin-Ansari et al., 2000)
ARNT	Mm.4316	F: ACTTTGTCAAGCTCATTTCC R: TGCAGCGAACTTTATTGATG	(Naruse et al., 2002)	Hs.166172	F: GCTGCTGCCTACCCTAGTCTCA R: GCTGCTCGTGTCTGGAATTGT	(Lin et al., 2003)
Bex1/Rex3	Mm.14768	F: CCAGGGAAGGATGAGAGA R: TAGAAGCTGGTAACAGGGAG	(Brown and Kay, 1999)	Hs.334370	F: ACAGGCAAGGATGAGAGAAG R: CCCACGTAAACAAGTGACAG	This laboratory
comesodermin	Mm.200692	F: GAGCCCTCAAAGACCCAGA R: CTAGGGACTTGTGTAAAAAGC	(Kimura et al., 1999)	Hs.301704	F: AATATCGGTGTTTTGGTAGG R: GTCCTCAGGGGTCGGAGC	(Kimura et al., 1999)
Hand1	Mm.4746	F: GGATGCACAAGCAGGTGAC R: CACTGGTTTAGCTCCAGCG	(Thattaliyath et al., 2002)	Hs.152531	F: TGCCTGAGAAAGAGAACCAG R: ATGGCAGGATGAACAAACAC	(Knofler et al., 2002)
HEB	Mm.36894	F: CCATCCCCAAATTCTGACGAT R: GCTGGCTCATCCCATTCG	(Ortman et al., 2002)	Hs.21704	F: ACTGAAAACAAAGAAAAGGATGAAAACC R:CCCTTTCTATCTTCTGTTCAGGGTTC	(Knofler et al., 2002)
mash2	Mm.196417	F: CAATAAAGATGACCTCTGTCCC R: GAATAGTACACTTTGCAACAGC	This laboratory	Hs.1619	F: CTCGCCCTCCCGCGGTTCTT R: CCAGCAGTGTCCCTCCAGCAG	(Westerman et al., 2001)
gp130	Mm.4364	F: CAGCACCAAGGATTTGGCTAGC R: GAAGTGCCATGCTTTGACTGGC	(Spence et al., 2001)	Hs.82065	F: GGTACGAATGGCAGCATACA R: CTGGACTGGATTCATGCTGA	(Sherwin et al., 2002)
LIFR	Mm.3174	F: CGGAATTCCTGACATATCCCAGAAGACACT R: GCTCTAGAGCATCTGTGGCTTATAGCCT	(Ni et al., 2002)	Hs2758	F: CTGGAACAGGCCGTGGTAC R: ACTCCACTCTTCGAGACCAG	(Knight et al., 1999)
ubiquitin C	Mm.331	F: AAGACCATCACCTTGGACG R: CCAAGAACAAGCACAAGGAG	This laboratory	Hs.183704	F: CCCAGTGACACCATCGAGAATG R: AACTTAGACACCCCCCCTCAAG	This laboratory
p16ink4a	Mm.4733	F: CATAGCTTCAGCTCAAGCAC R: CAATCCAGCCATTATTCCCTTC	This laboratory	Hs.1174	F: CACCGCTTCTGCCTTTTCAC R: AGCTTTGGTTCTGCCATTTGC	This laboratory
cyclin D2	Mm.3141	F: AGCTTCCAAGCTGAAAGAGACC R: CAACACTACCAGTTCCCACTCC	This laboratory	Hs.75586	F: TGTGAGGAACAGAAGTGCGAAG R: AACATGCAGACAGCACCCAG	This laboratory
mdm2	Mm.22670	F: GAATCCTCCCCTTCCATCACAC R: AAGCCTTCTTCTGCCTGAGC	This laboratory	Hs.170027	F: AGCAGGAATCATCGGACTCAGG R: CACACAGAGCCAGGCTTTCATC	This laboratory
GADD45	Mm.1236	F: GTTACTCAAGCAGTCACTCCCC R: TCTTCAGGCTCACCTCTCTCTC	This laboratory	Hs.80409	F: TTGTTTTTGCCGGGAAAGTCG R: TTGAACTCACTCAGCCCCTTG	This laboratory
TNFR2	Mm.1258	F: GAACAATTCCATCTGCTGCACC R: TTACAGCCACACACCGTGTC	This laboratory	Hs.256278	F: TGGACTGATTGTGGGTGTGAC R: TTATCGGCAGGCAAGTGAGG	This laboratory
TRAF1	Mm.12898	F: TGCAGAGCAGACAACCTCCATC R: TCCCCTTGAAGGAACAGCCAAC	This laboratory	Hs.2134	F: TGTACCTGAATGGAGATGGCAC R: TCTGGTCCAGCAGCATGAAG	This laboratory
CSF1	Mm.795	F: ACATCCACCACTACCCTCTC R: ACCCCATCAAAGCTGCTTC	This laboratory	Hs.173894	F: CCCTCCTCCAAACTTCAACTCC R: ATCCTCTGACCTCCCCTGAATC	This laboratory
FGF11	Mm.269011	F: ATCCCGATGGGAGTATCCAG R: GCAGAGGCATACAGGACATAG	This laboratory	Hs.31339	F: GCTCCTTCACCCACTTCAAC R: GGCGTACAGGACGTAGTAATTC	This laboratory
IL19	Mm.131480	F: CGCCTCATAGAAAAGAGTTTCC R: GCAATGCTGCTGATTCTCC	This laboratory	Hs.71979	F: CAAGAAATCAAAAGAGCCATCC R: CTCTGTTCCTGACATTGCC	This laboratory

onto glass coverslips coated with gelatin or used for RNA purification.

Gene detection by RT-PCR

Total RNA was isolated from cell pellets using RNAeasy Qiagen mini protocol. cDNA was synthesized using 100 ng to 1 μg of total RNA in a 20-μl reaction. Superscript II (Invitrogen), a modified Maloney murine leukemia virus RT, and Oligo (dT) $_{12-18}$ primers were used according to the manufacturer's instructions. Aliquots of cDNA, equivalent to 1/20 of the above reaction, were used in a 20-μl reaction volume. PCR amplification of different genes was performed using RedTaq DNA polymerase (Sigma). PCR reactions were run for 35 cycles (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min) and incubated at 72°C for 10 min at the end to ensure complete extension. The PCR products were subjected to electrophoresis in 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. One hundred-basepair DNA ladder (Invitrogen; catalog # 10380-012) was used to estimate size of the amplified bands. Table 1 lists the primers used for RT-PCR.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 20 min at room temperature. Fixed cells were blocked for 1 h at room temperature with PBS/0.1% BSA/10% normal goat serum/0.3% Triton X-100 and then incubated overnight with primary antibodies in PBS/0.1% BSA/10% normal goat serum at 4°C. The list of primary antibodies and their working dilutions are shown in Table 2. After three washes in PBS/0.1% BSA, fluorescent secondary antibodies (Molecular Probes and Jackson ImmunoResearch) at 1:200 dilution in PBS were incubated with cells for 1 h at room temperature to detect expression. Double labeling experiments were performed by simultaneously incubating cells in appropriate combinations of primary antibodies, followed by incubation with noncross-reactive secondary antibodies. Staining with antibodies against the cell surface markers E-NCAM, A2B5 was carried out in cultures of living cells without blocking in culture medium for 1 h at 37°C.

Staining for alkaline phosphatase was performed with a kit (Sigma 85L1-1-KT) according to the manufacturer's protocol.

Electron microscopy

Undifferentiated H1 and D3 were grown on a MEF feeders. Once they formed sizable colonies, they were fixed with 5% glutaraldehyde for 3 h and processed for electron microscopy. They were postfixed in osmium tetroxide, embedded in plastic, semi-thin sectioned at 1 μm, and stained with toluidine blue. In addition, thin sections were prepared for electron microscopy according to standard protocols (Wood et al., 1990).

Table 2
Antibodies used for immunofluorescence

Epitope name	Clone	Dilution	Description	Source
A2B5	A2B5	1:20	Mouse IgM	ATCC
BCRP1	5D3	1:200	Mouse IgG2b	Chemicon
CD44	Hermes-3	1:25	Mouse IgG	Sherman et al., 2000
GFAP	–	1:500	Rabbit	DAKO
ENCAM	5A5	1:5	Mouse IgM	DSHB
Nestin (anti-human)	Nestin 331B	1:2000	Rabbit	Messam et al., 2000
Nestin	Rat401	1:5	Mouse IgG1	DSHB
β-III tubulin	SDL.3D10	1:2000	Mouse IgG2b	Sigma
β-III tubulin	the same as TUJ-1	1:2000	Rabbit	Covance
CD15S; anti-sialyl Le^X	CSLEX1	1:200	Mouse IgM	BD
Connexin43	–	1:1000	Rabbit	Chemicon
FGFR-4	137105	1:20	Mouse IgG	R & D Systems
FGFR-4	VBS1	1:250	Mouse IgM	Chemicon
HNF3-beta	4C7	1:50	Mouse IgG1	DSHB
Islet1	394D5	1:50	Mouse IgG2b	DSHB
Notch-1	BTAN20	1:50	Rat IgG	DSHB
OCT3/4	N-19	1:100	Goat	Santa Cruz
Sox-2	–	1:2000	Rabbit	Dr. L. Pevny, (Kamachi et al., 1995)
Sox-2	–	1:2000	Rabbit	Chemicon
SOX-2	–	1:200	Rabbit	Abcam
SSEA-1	MC-480	1:50	Mouse IgM	DSHB
SSEA-3	MC-631	1:50	Rat IgM	DSHB
SSEA-4	MC-813-70	1:50	Mouse IgG3	DSHB
TRA-1-60	TRA-1-60	1:100	Mouse IgM	Chemicon
TRA-1-81	TRA-1-81	1:100	Mouse IgM	Chemicon
TRA-2-10	TRA-2-10	1:50	Mouse IgG1	DSHB
Vimentin	V9	1:50	Mouse IgG1	Sigma

cDNA microarrays

The nonradioactive GEArray™ Q series cDNA expression array filters for human and mouse cell cycle, apoptosis and cytokine genes (Cat #: HS-001N, HS-002N, HS-003N and MM-001N, MM-002N, MM-003N, respectively, SuperArray Inc.) were used according to the manufacturer's protocol. Total RNA was isolated from cell pellets using RNAeasy Qiagen mini protocol and kit. The biotin dUTP labeled cDNA probes were specifically generated in the presence of a designed set of gene-specific primers using total RNA (4 mg per filter) and 200 U MMLV reverse transcriptase (Promega). The array filters were hybridized with biotin-labeled probes at 60°C for 17 h. After that, the filters were first washed twice with 2 × SSC/1% SDS and then twice with 0.1 × SSC/1% SDS at 60 °C for 15 min each. Chemiluminescent detection was performed by subsequent incubation of the filters with alkaline phosphatase-conjugated streptavidin and CDP-Star substrate. Array membranes were exposed to X-ray film. Quantification of the gene expression was performed with ScionImage software. Mode OD of each gene/spot was calculated and normalized to expression of GAPDH. Human and mouse

genes and OD values were aligned. Some human genes had no counterparts on mouse arrays and vice versa. cDNA microarray experiments were done twice with new filters and RNA isolated at different times.

Telomerase activity

Telomerase was measured using the telomeric repeat amplification protocol (TRAP) assay as described (Kim et al., 1994; Weinrich et al., 1997), Terminal restriction fragment (TRF) size was determined using Southern hybridization as described (Allsopp et al., 1992; Harley et al., 1990).

EST-enumeration

EST frequency counts of genes expressed in human ES cells were performed as described (Brandenberger et al., submitted for publication). Briefly, cDNA libraries of human ES cell lines H1, H7, and H9 grown in feeder-free conditions, embryoid bodies (EB), and two differentiated subpopulations were constructed and submitted for EST sequencing. The EST sequences were assembled into overlapping sequence assemblies and mapped to the UniGene database of nonredundant human transcripts. Expression levels were assessed by counting the number of ESTs for a particular gene that were derived from the undifferentiated human ES cells and comparing them to the number of ESTs derived from the differentiated subpopulations. Statistical significance was determined using the Fisher Exact Test (Siegel and Castellan, 1988). Expression levels are presented as number of EST expressed in the undifferentiated human ES cells, and embryoid bodies (EB), for example, for Oct-4 [ES:EB], the numbers are [24:1] indicating that there are 24 ESTs encoding Oct-4 found in the undifferentiated human ES cells and 1 EST in EBs.

Results

Assessment of human and mouse ES cell cultures based on morphology and on expression of commonly used markers

Undifferentiated mouse and human ES cell were cultured and assessed by electron microscopy, immunocytochemistry and RT-PCR. Many similarities and numerous differences between the undifferentiated human H1 and mouse D3 cells were noted. On electron microscopy examination (Fig. 1). H1 cells formed a 2- to 4-cell layer over the feeder cells. In contrast, D3 cells (Fig. 1) formed aggregates with 4–10 cell layer thickness over the feeder layer. Phase contrast photographs (see Fig. 2A panels a and b) demonstrated that human ES cells unlike mouse ES cells formed round colonies with well-defined edges, a pattern similar to find-

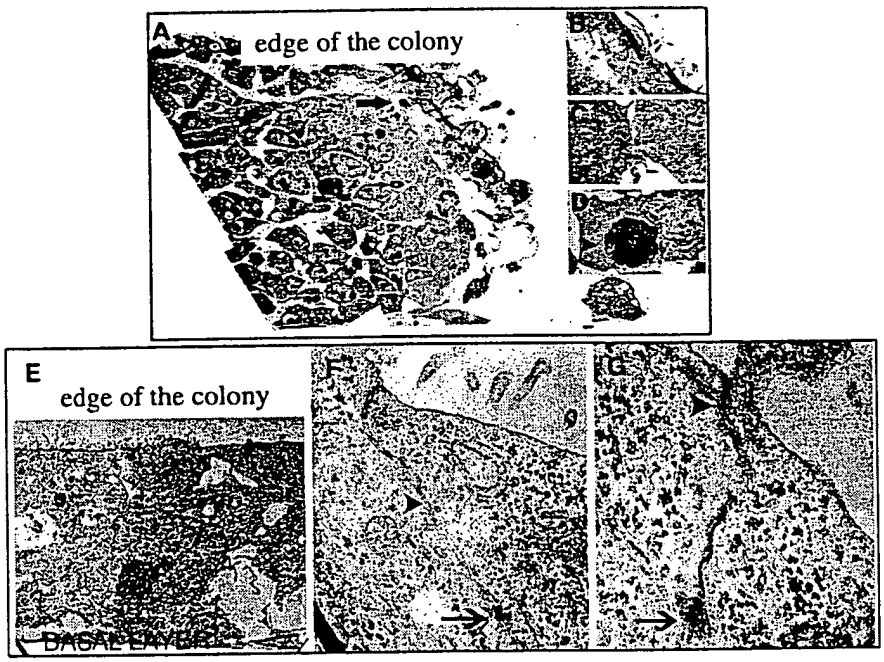


Fig. 1. Electron microscopy of undifferentiated mouse ES and human ES cells: (A–D) Mouse D3 cells. (A) Composite of nine fields taken at 2,000× magnification. Small arrows point to autophagosomes and the large arrow points to an apoptotic body. (B) Cells at the colony edge often had microvillae (original magnification 20,000×). (C) Tight junctions (arrow) and gap junctions (arrowheads) can be seen (original magnification 17,000×). (D) A higher magnification view of the autophagosome (original magnification 10,000×). (E–G) Human H1 cells. (E) A low power (2000×) electron micrograph. (F and G) Tight junctions (arrow) and gap junctions (arrowheads) can be seen (original magnification 20,000× and 30,000× for F and G, respectively).

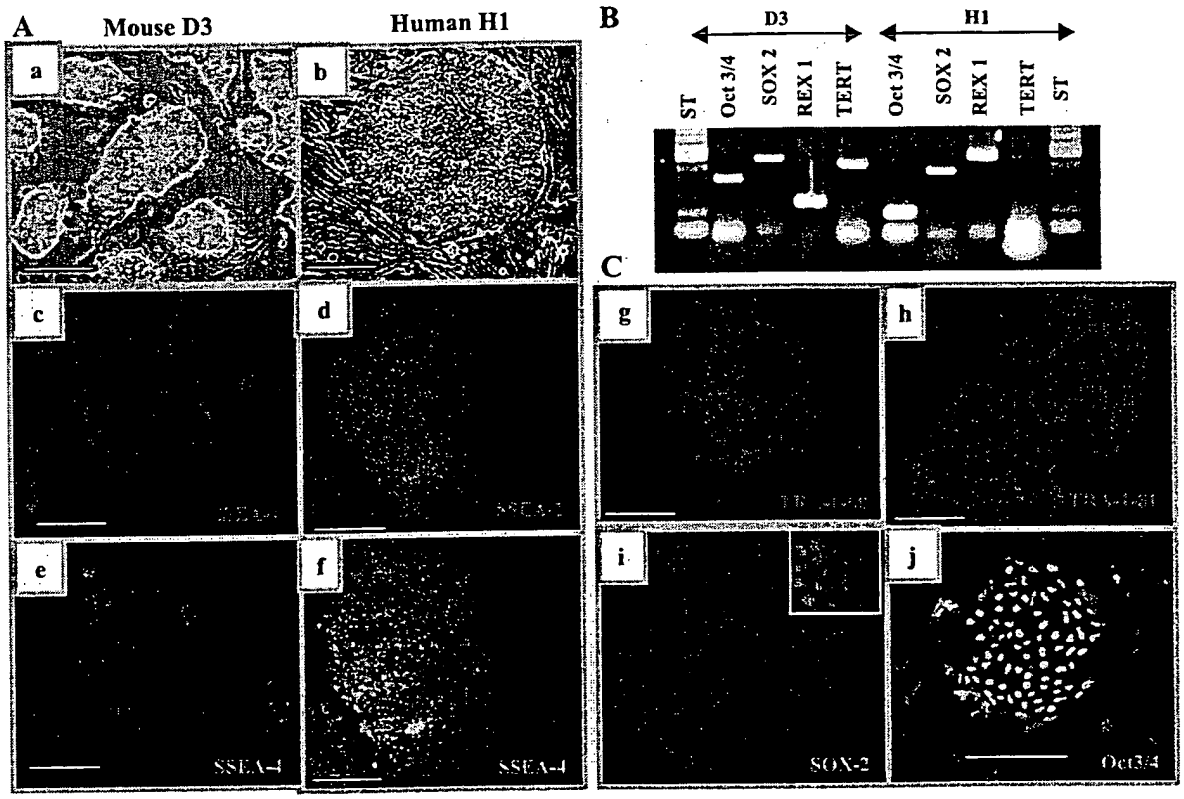


Fig. 2. Characterization of undifferentiated state of H1 human and D3 mouse ES cells with commonly used markers. (A) Immunostaining. Panels a and b are phase contrast microphotographs of live H1 and D3 cultures. Unlike D3 cells, H1 cells form round colonies with well-defined edges. (c,d) SSEA-1 staining; (e,f) SSEA-4; (B) RT-PCR results. Genes previously reported to be associated with pluripotent state (OCT3/4, SOX-2; REX-1 and TERT) are expressed in both types of cells. All experiments performed at least twice. (C) expression of TRA-1-60 (g) and TRA-1-81 (h) SOX-2 (i) and OCT3/4 (j) in H1 cells. Scale bars = 200 μm.

ings by other labs. Both the H1 and D3 cells had a large nucleus-to-cytoplasm ratio with numerous ribosomes. Both ES cell colonies had a clearly polarized structure with the ES cells harboring numerous villae facing the feeding medium. There were tight junctions and gap junctions among cells in both ES cell lines, especially close to the surface (Figs. 1C, D, F, and G for mouse and human cells, respectively). These findings were consistent with our immunostaining for Connexin 43 (see Fig. 3). No highly organized intermediate filamentous structures, such as neurofilaments, that are suggestive of differentiation were seen. Small aggregates of microtubular bundles ran in the perimeter of the nuclei. One finding that clearly segregated D3 cells from H1 cells was the presence of many apoptotic cells, especially close to the edge of the colony, and autophagosomes in almost a quarter of all of the mouse ES cells. These electron dense lysosomal structures were rare in human ES cells.

The quality of D3 and H1 cells cultured in our laboratory was confirmed by evaluating the expression of several markers that are expressed by undifferentiated ES cells (Carpenter et al., 2001; Reubinoff et al., 2000; Thomson et al., 1998a). Mouse D3 cells expressed SSEA-1 but did not express SSEA-4, while H1 cells exhibited reciprocal expression of these stage-specific embryonic antigens (Fig. 2). Both cell lines expressed SSEA-3, although SSEA-3-positive cells were less abundant in mouse cultures (data not shown). In addition, H1 cells (but not D3 cells) expressed TRA-1-60 and TRA-1-81 antigens (data for D3 cells not shown).

RT-PCR was performed to assess expression of genes characteristic for ES cells such as OCT3/4 (Okamoto et al., 1990; Rosner et al., 1990), SOX-2 (Avilion et al., 2003; Cai et al., 2002), BEX/REX-1 (Ben-Shushan et al., 1998; Rosfjord and Rizzino, 1994), and TERT (Armstrong et al., 2000; Niida et al., 1998). Bands of the appropriate size were observed for all these genes using species-specific primers (Fig. 2), confirming that the cells grown in our laboratory appeared similar to previously described undifferentiated ES cells. OCT3/4 and SOX-2 protein expression was confirmed with immunostaining. Most D3 and H1 cells were positive for SOX-2 and Oct 3/4 (Fig. 2 and data not shown). Overall, these results demonstrate that D3 and H1 cells were not different significantly in morphology, antigen immunostaining, and pluripotency marker expression when compared to reports from other laboratories but differed from each other.

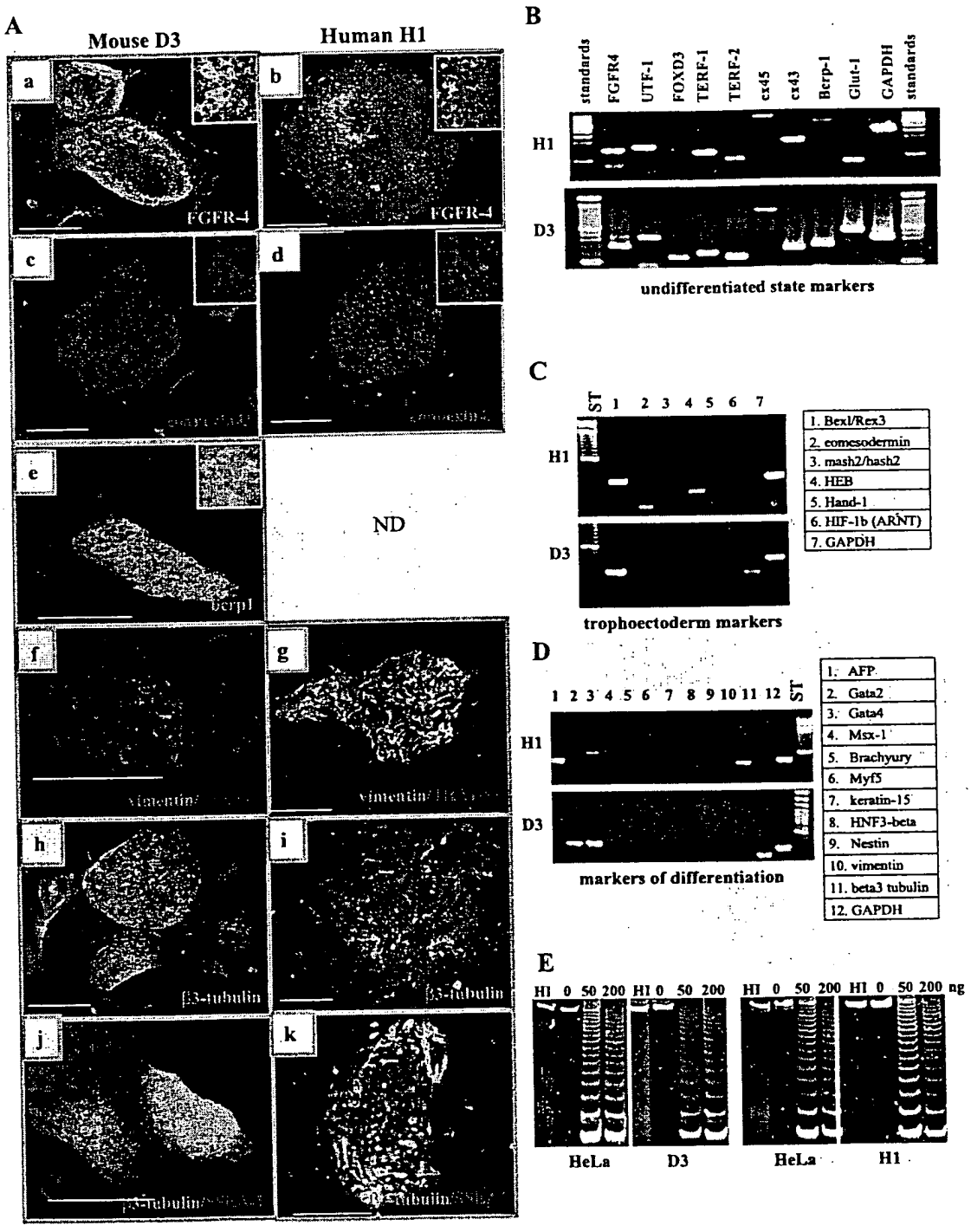


Fig. 3. Characterization of undifferentiated state of H1 human and D3 mouse ES cells with an expanded set markers. (A) Results of immunostaining. Panels a, c, e, f, h, j, mouse cells; panels b, d, g, i, k, human cells. (a,b) FGFR-4; (c,d) connexin-43 (all insets-membrane localization); (e) BCRP1 mouse cells; (f) double staining for vimentin and undifferentiated state marker SSEA-1 (g) human cells; double staining for vimentin and undifferentiated state marker TRA-1-81. (h,i) beta-tubulin class III in mouse and human cells, respectively, (j,k) double staining for beta-tubulin class III and for SSEA-1 and SSEA-4 in mouse and human cells, respectively. All photographs are taken under 20× magnification; scale bars = 200 μm; ND: not detected. (B) RT-PCR analysis of additional markers of undifferentiated state. (C) RT-PCR analysis of trophoectoderm markers. ST-standard 100 kb DNA ladder. (D) RT-PCR analysis of germ layer markers. Only positive results are presented. (E) Comparison of telomerase activity. Different amounts (0, 50, 200 ng) of cell extracts from HeLa (positive control), D3 and H1 cell lines were assayed for telomerase activity by TRAP assay. HI-heat-inactivated extract (1000 ng) was used as a negative control.

Additional markers that serve to characterize mouse and human ES cell lines

To expand the list of markers expressed in undifferentiated cells, we tested expression of additional markers of pluripotency that are thought to be controlled by OCT3/4 and SOX-2 genes, such as UTF-1 (Nishimoto et al., 1999; Okuda et al., 1998), FOXD3 (Hanna et al., 2002) and FGFR-4 (McDonald and Heath, 1994; Niswander and Martin, 1992; Wilder et al., 1997) and telomerase-associated factors TERF1 and TERF2 (Broccoli et al., 1997; Karlseder et al., 1999; van Steensel and de Lange, 1997). RT-PCR amplification of transcripts of all these genes except FOXD3 demonstrate their presence in both D3 and H1 cells (Fig. 2B). Expression of FOXD3 was detected only in mouse cells. Immunostaining confirmed FGFR-4 protein expression on the cell surface of both human and mouse cells (Fig. 3A (a) and (b)). Expression of TERT, TERF1, and TERF2 genes in both mouse ES and human ES was consistent with high telomerase activity measured with TRAP assay. Telomerase activity in both ES cell lines was as high as in HeLa tumor cells (Fig. 3E).

We also tested the expression other cell surface markers reported to be present on blastocysts or other stem cell populations, such as gap junction proteins connexins-43 (Duval et al., 2002) and 45 (Rozental et al., 2000), glucose transporter GLUT1 (Leppens-Luisier et al., 2001; Morita et al., 1994; Pantaleon et al., 2001), and ABC transporter BCRP-1/ABCG-1 (Cai et al., 2002; Lu et al., 2002; Zhou et al., 2002, 2001). All of these genes could be readily detected in both H1 and D3 cells (Fig. 3B). All except GLUT-1 were absent in feeder cells (data not shown), making these markers useful in distinguishing ES cells from contaminating feeder populations. Connexin 43 expression was confirmed by immunostaining in both mouse and human cells (Fig. 3A(c) and (d)). The antibody against BCRP1 recognized only the mouse epitope in our experiments (Fig. 3A(e)). The results of the RT-PCR and immunostaining for stem cell-specific markers are summarized in Table 3A. Markers detected in both mouse and human ES cells (black boxes) or unique to human ES cells (gray boxes) are highlighted.

Markers of differentiated phenotypes

Human ES cells, like nonhuman primate ES cells (but unlike mouse ES cells) are able to differentiate into trophoblast in culture (Odorico et al., 2001; Thomson et al., 1998a). To assess the expression of trophoblast-related genes in H1 and D3 cells, we used published RT-PCR primers for early trophoectoderm markers to analyze their expression. Genes tested included Bex1/Rex3 (Williams et al., 2002), eomesodermin (Russ et al., 2000), and four other transcription factors: Mash2/Hash2, HEB, Hand-2 and HIF-1beta/ARNT, all described by Janatpour et al. (1999). As shown in Fig. 3C, both mouse D3 and H1 cells expressed BEX1/REX3. In addition, D3, but not H1 cells, expressed

Table 3

Gene	H1		D3	
	RT-PCR	staining	RT-PCR	staining
(A)				
OCT 3/4	yes		yes	
SOX-2	yes	yes	yes	yes
UTF-1	yes		yes	
REX-1	yes		yes	
TERT	yes		yes	
TRF1	yes		yes	
TRF2	yes		yes	
FOXD3	no		yes	
Cx 43	yes	yes	yes	yes
Cx 45	yes		yes	
FGFR-4	yes	yes	yes	yes
ABCG-2	yes		yes	
Glut-1	yes		yes	
SSEA-1		no		yes
SSEA-4		yes		no
SSEA-3		yes		yes
TRA-1-81		yes		no
TRA1-60		yes		no
(B)				
Bex1	yes		yes	
emsd	yes		no	
HEB	yes		no	
mash2	no		no	
Hand1	yes		no	
HIF-1b	no		yes	
(C)				
Actc1	no		no	
Brachyury	no		no	
Msx-1	no		no	
Myf 5	no		no	
Gata4	yes		yes	
AEP	yes		no	
Islet-1	no	no	no	no
Pdx1	no		no	
HNF3-b	no	no	no	no
Krt1-14	yes		no	
Krt1-15	no		no	
Brain-1	no		no	
Nestin	no	no	no	no
vimentin	yes	yes	no	no
betaIII-tubulin	no	no	yes	yes
CD44		no		no
GFAP		no		no
PS-NCAM		no		no
A2B5		no		no
TRA2-10		no		no
Notch-1		no		no
CD15s		ND		no

Gene expression in human H1 and mouse D3 ES cells was analyzed using RT-PCR and immunostaining. Representative (mainly positive) results of these experiments are presented in Figs. 1 and 2. Antibodies and primer sequences are listed in Tables 1 and 2, respectively. (A) markers of undifferentiated state; (B) trophoectoderm markers; (C) markers of germlayer differentiation. Black boxes—commonly expressed markers; white boxes—markers are not expressed in both H1 and D3 cells; gray boxes—markers are differently expressed in H1 and D3 cells.

aryl hydrocarbon receptor nuclear translocator (ARNT) (Abbott and Probst, 1995; Jain et al., 1998). Among other trophoectoderm markers tested, eomesodermin was persistently expressed in all human ES cell samples and HEB transcripts appeared in some but not all samples (data not shown). Mash2 and its human homolog Hash2 were also present in some RNA preparations from both cell types (Fig. 2C). The results summarized in Table 3B suggest trophoectoderm-specific markers are differentially expressed in human and mouse cells. In accordance with the RT-PCR results, EST frequency counts for trophoectoderm markers suggested high expression of HEB in both, undifferentiated cells and embryoid bodies [7:12], while BEX1 [0:1] and HAND2 [0:1] were expressed only in embryoid bodies. ARNT was not detected in human ES cells.

Besides identification of a set of positive ES cell-specific markers, we also sought to develop a set of negative markers to assess the state of differentiation of current ES cell lines. Genes that have been previously reported as markers of early differentiation into germ layers or into tissue-specific precursors were chosen as sensitive indicators of differentiation. Results are summarized in Table 3C. Several markers that were thought to be characteristic of differentiation were expressed by undifferentiated cultures of human and in mouse ES cells. Thus, GATA4, a zinc finger transcription factor, thought to be important for early endoderm/heart differentiation was present in both mouse and human ES cell lines, although cardiac actinin was not expressed (Fig. 3D and Table 3C). EST frequency analysis confirmed expression of GATA4 (and detected no actinin) in undifferentiated cells and in embryoid bodies [3:3]. Mouse D3 cells expressed GATA2, which was not detected in undifferentiated human ES cells using RT-PCR and EST counts [0:1]. Another marker of visceral endoderm, alpha-fetoprotein (AFP) was present in H1 cells (Fig. 3D and Table 3C), although not detected by EST scan. Immunos-

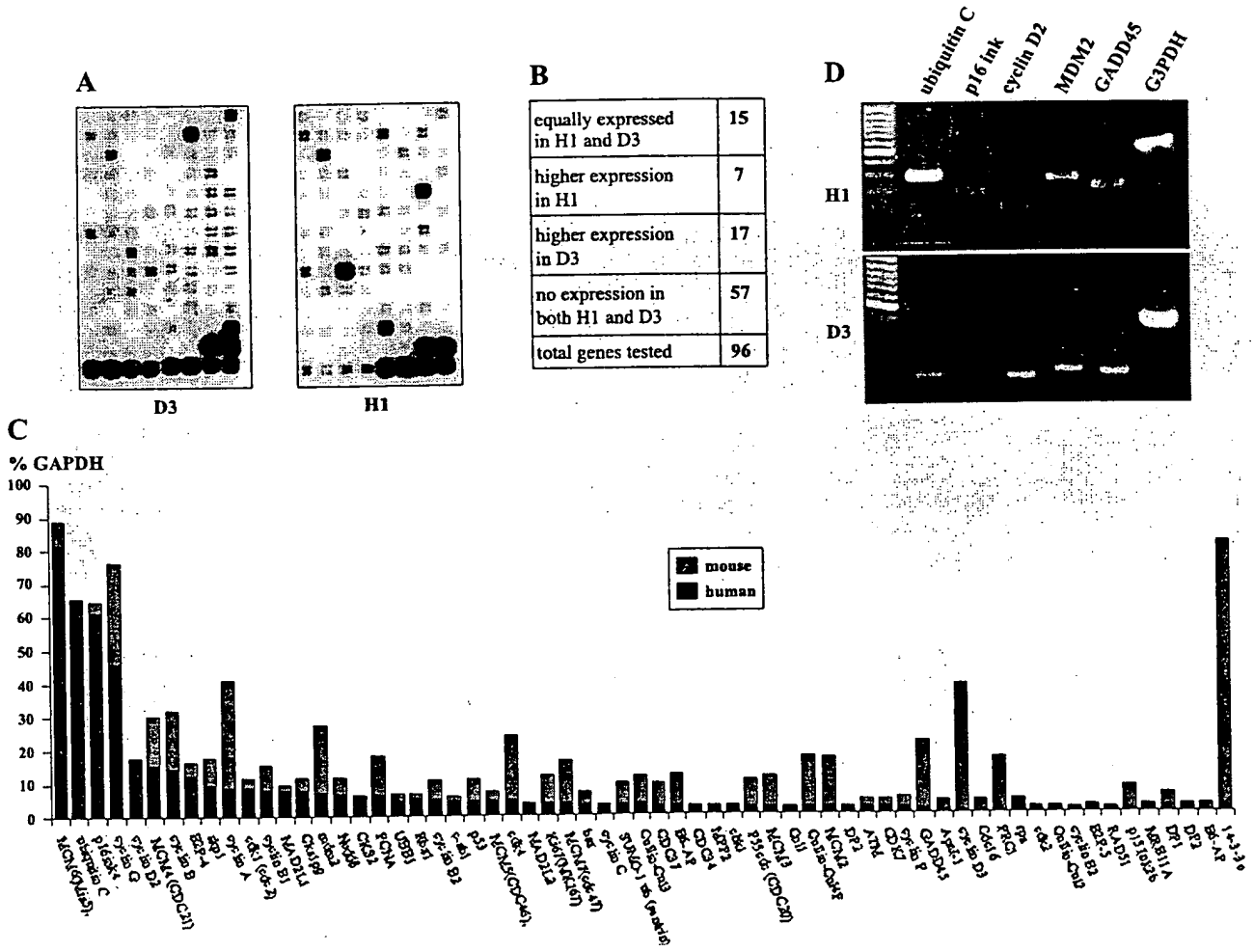


Fig. 4. Comparison of cell cycle-related gene expression in undifferentiated D3 mouse and H1 human ES cells using cDNA microarrays. (A) Representative hybridization result is shown. (B) General comparison of the array results. (C) Quantification of the gene expression with ScionImage software. Each bar represents a mean of two separate experiments for each cell type. Only genes with expression of > 2% GAPDH are presented. The full list of genes and OD values is available upon request. (D) RT-PCR verification of cell cycle microarray results for several genes.

taining studies also confirmed the presence of differentiation markers in both D3 and H1 cells. For instance, significant number of human H1 cells expressed vimentin. Double staining for vimentin and for a human marker of undifferentiated state TRA-1-81 showed no co-localization, suggesting that vimentin-positive cells were a differentiated cell population (Fig. 3A(g)). Previous studies of human cell ES lines H7 and H9 cultured on Matrigel (Carpenter et al., 2001) demonstrated expression of neuronal precursor marker beta-tubulin class III in these cultures and was confirmed using EST frequency analysis for undifferentiated cells and embryoid bodies [2:8]. When the presence of beta-tubulin class III was tested with immunostaining in our cultures, human H1 cells were mostly beta-tubulin class III-negative, except at the edge of the colony, where expression of SSEA-4 antigen has been lost (Fig. 3A(i) and (k)). However, beta-tubulin class III was found in mouse D3 cell cultures, where its expression co-localized with the mouse marker of undifferentiated cells, SSEA-1 (Fig. 3A(h) and (j)). On the other hand, no vimentin was found in mouse ES cells (Fig. 3A(f)). Patterns of expression of beta-tubulin class III and vimentin in D3 and H1 cells were confirmed with RT-PCR (Fig. 3D). Mouse ES cells were grown without feeders for RNA purification and did not express keratin 14 (Fig. 3D), neither was it expressed in human cell ES lines H1, H7, and H9 cultured on Matrigel without feeders (see Fig. 8B).

In summary, broader analysis of stem cell markers identified additional partially overlapping marker sets markers, which could be used to assess the state of human and mouse ES cell cultures.

Comparison of cell proliferation and cell death gene expression in D3 and H1 ES cell lines

Given the differences observed by immunocytochemistry and by RT-PCR in marker and gene expression, it was reasoned that human and mouse cells will display additional differences in their cell cycle regulation and cytokine response. To directly test this, we employed focused microarrays containing 96 genes related to cell cycle or apoptosis as previously described (Luo et al., 2002). To avoid a bias in hybridization, arrays containing human genes were used to probe expression in human cells, and homologous arrays of mouse genes (80 out of 96 genes were homologous) were used to assess expression in mouse cells. Relative OD of each spot on the array was quantified with ScionImage and normalized to GAPDH. All numbers below are percentages of GAPDH expression. The results of the cell cycle microarray are summarized in Fig. 4. As can be seen in Figs. 4A and B, almost half of all genes on each array was expressed, providing evidence for cell proliferation in both types of ES cell cultures. Eleven of the mismatched genes were present at low or undetectable levels. Three genes which were absent on the mouse array and present on the human array were expressed at detectable levels: CDC28 protein kinase 2 (UniGene: Hs.83758; 6.4%; EST counts [3:0]; MAD2 (UniGene; Hs.19400; 3.97%; EST counts [6:3]); and CDC28 protein kinase 2 (UniGene Hs.83758; 6.42%). Mouse cells expressed two genes that were absent on the human array: E2F-related transcription factor (UniGene Mm.925; 6.13%) and p53-activated gene 14-3-3e or tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (UniGene Mm.42972; 80.87%).

Table 4
Comparison of the results of the cell cycle array with EST counts

Cell cycle	Higher in D3	Percentage array control[a] (%)	Estimated frequency[b]
G1	Cyclin D3	0.9	1
	CDK4	4.6	7
	p15	8.23	ND
	Cullin 3	3.4	1
	Cullin 4B	2.4	4
S	MCM2	2.4	23
	MCM3	2.4	38
	MCM7	3.7	19
	Ki67	4	0
G2	Cyclin F	1.8	1
M	P55 CDC20	2.4	7
	PRC1	0.9	6
p53/DNA damage	GADD45A	4.3	1
	14-3-3e	ND	ND
	SUMO-1	3.4	5
	UBE3A	3.1	2
	RPA3	0.9	0
Cell cycle	**Equal in D3 and H1**	**Percentage array control[a] (%)**	**Frequency counts[b]**
G1	Cyclin E1	8	0
	CKS1	7.6	3
	NEDD8	6.7	3
	SKP1A	9.5	5
	E2F4	12.2	3
S	Cyclin A	8.9	3
	Cyclin G1	46	4
	PCNA	6.4	10
	MCM4	15.6	34
	MCM5	4.6	13
G2	Cyclin B1	14	8
	Cyclin B2	5	6
M	RBX1	5.5	0
P53 DNA damage	p53	4.6	4
	MDM2	7.3	0
Cell cycle	**Higher in H1**	**Percentage array control[a] (%)**	**Frequency counts[b]**
G1	Cyclin D2	18	5
	p16ink	61	ND
	CKS2	6.4	3
S	MCM6	82	12
G2	None		
M	None		
p53 DNA damage	cAbl	4.6	ND
	UB-C	65.5	0
	UB-E1	6.1	44

[a] %GAPDH expression (OD) on the array.
[b] Results of EST scan; maximum detected frequency count was 44.

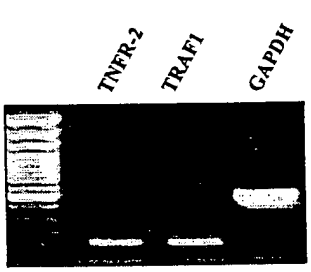
Similarities and differences in expression of some key cell cycle regulators are summarized and compared to EST frequency scan for the same genes (see Table 4). Good correlation between two methods was observed though a few genes, which were not detected by EST scan (ND), were expressed at low levels as assessed by array analysis. In addition, the expression of two genes not present on the array and elevated in mouse ES cells were confirmed as being expressed E2F [2:2] and tyrosine 3-monooxygenase [20:21].

As one would expect, markers of proliferation Ki67, PCNA, and cyclins were expressed, although with different patterns in each cell type. Expression of tumor suppressor genes such as ATM, Rb and retinoblastoma-like proteins, and of BRCA1 was not detected. Expression of p53 was low in human cells, but was higher in mouse cells (4.2% vs. 7.5%, respectively). Negative regulators of the p53 and Rb pathway such as mdm2 were present as well. Cell cycle inhibitors p21, p27, p18, and p19 were undetectable by hybridization, while p16 inhibitor was predominantly expressed in H1 cells (60.9% vs. 3.73%). Moderate expression of p15 inhibitor (8.3%) was observed in mouse cells only. Mouse cells also showed moderate expression (5.8%) of one of the DNA-binding replication proteins, RPA3 (Unigene: Mm.29073), which was absent in human cells.

The ubiquitination pathway appeared more active in H1 cells as ubiquitin C was virtually undetectable in mouse D3 cells but was present at high levels (65.5%) in human cells. GADD45 was readily detectable in the mouse D3 line (20.1%) but was low or absent in human cells (1.5%). MCM genes 2 through 7, which are thought to be "DNA licensing factors", which bind to the DNA after mitosis and enable DNA replication before being removed during S phase (Lei and Tye, 2001; Nishitani and Lygerou, 2002), were differentially expressed as well. MCM-6 was abundantly expressed in human H1 cells (81.7%; EST counts [12:5]), while MCM-2, MCM-3, and MCM-7 expression was more prominent in D3 cells (14.8%, 8.7% 12.7%, respectively), although EST analysis detected quite high levels of MCM-2 [23:8] and MCM-7 [19:3], but not MCM-3 [1:0]. MCM-4 and MCM-5 were equally expressed (around 13–15%; EST counts [34:9] and [13:3], respectively). Expression of selected genes (ubiquitin C, p16ink, cyclin D2, mdm2, and GADD45) was confirmed with RT-PCR. Similar to the microarray results, ubiquitin C and CDK4 inhibitor p16ink were expressed at higher levels in

A

Equally expressed in H1 and D3	2	MCL, BAX
Higher expression in H1	15	mdm2, TNFR2, TRAF6, RIP2, caspase14, caspase3, caspase6, bcl-x, blk, bak, HRK, chk1, p63, Bruce
Higher expression in D3	7	p53, GACC45, survirin, DR3, DR6, rpa, bik
No expression in both H1 and D3	72	
Total genes tested	96	



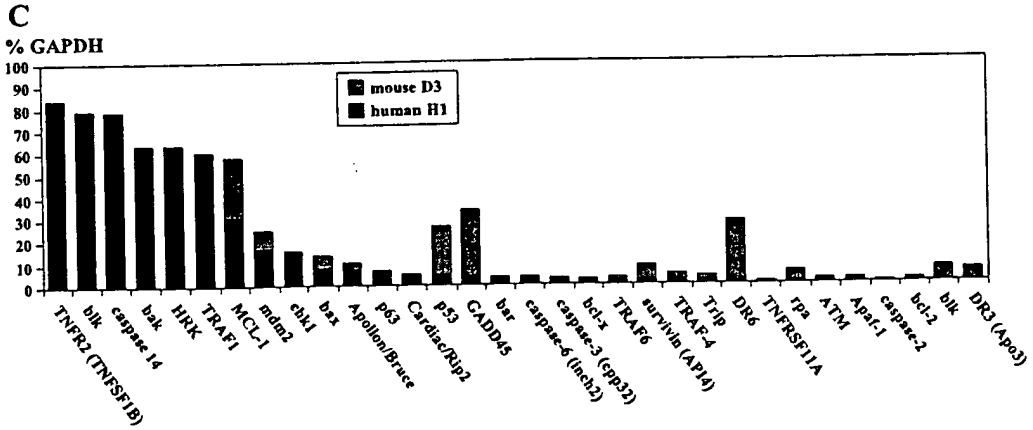


Fig. 5. Comparison of apoptosis-related gene expression in undifferentiated D3 mouse and H1 human ES cells using cDNA microarrays. (A) General comparison of the array results. (B) RT-PCR confirmation of TNFR-2 and TRAF1 expression in human cells. (C) Quantification of the gene expression with ScionImage software. Mode OD of each gene/spot was calculated and normalized to GAPDH expression. Each bar represents a mean of two separate experiments for each cell type. Only genes with expression of >2% GAPDH are presented. The full list of genes and OD values is available upon request.

human ES cells, while other genes were expressed at similar levels (Fig. 4B).

Examining regulation of cell death pathways showed the presence of a limited subset of genes (Fig. 5). Nine and 17 genes were expressed at detectable levels in D3 and H1 cells, respectively (see Fig. 5A for the lists of these genes). Surprisingly, only 2 out of these 24 genes were expressed in both cell types. One was the anti-apoptotic BCL2-related gene MCL-1 (27.3% and 30.6% of for D3 and H1 cells, respectively); and the other was BAX (6.2% and 7.9% for D3 and H1, respectively), which causes cytochrome *c* release from mitochondria. Mouse, but not human cells, expressed additional BAX-related gene BIK (7.4% and 0% for D3 and H1, respectively). Human cells in contrast, expressed BAK (63.7%) and HRK (63.29%), other BAX-related genes.

Many of the genes that were present at detectable levels in undifferentiated mouse ES cell cultures were associated with the p53 pathway (Fig. 4B). Indeed, the p53 effecter GADD45 was highly expressed only in mouse ES cells (29.5%), and expression of another p53-dependent gene 14-3-3e (Mm.42972) was also high in D3 cells, although this gene had no counterpart in human apoptosis array. Human ES cells in contrast expressed higher levels of negative regulators of p53 function. These included mdm2 ubiquitin ligase, which binds p53 and destines it for ubiquitination and degradation (8.5% and 16.4% for D3 and H1 cells, respectively).

In contrast to mouse ES cells, human ES cells expressed several genes associated with TNF receptor signaling. This includes high levels of p75 TNFR-2 (84.5% and 0% for H1 and D3 cells, respectively) and TNF receptor-docking molecule TNF receptor-associated factor TRAF1 (60.0% and 0.3% for H1 and D3 cells, respectively). These results have been confirmed with RT-PCR (Fig. 5B), TRAF6, and TRAF4, as well as one of the members of the receptor-interacting family of Ser/Thr kinases RIP2 (5.3%) were also expressed predominantly in human ES cells, although to the lesser extent (RIP2 homologue was absent on the mouse array). Human ES cells demonstrated differentially high expression of a BLK gene (Hs.2243 and Mm.3962; 79.3% and 0%, respective-

A

Gene name	UniGene	H1 % GAPDH	UniGene	D3 % GAPDH
CSF-1	Hs.173894	67.5	Mm.795	2.1
IL19	Hs.71979	57.7	N/A	
FGF11	Hs.31339	22.8	Mm.57238	0.7
IL-2	Hs.89679	0.0	Mm.14190	121.1
TNFSF13B	Hs.270737	1.1	Mm.195095	104.2
fgf4	Hs.1755	1.4	Mm.4956	45.1
Kit ligand			Mm.4235	31.7
IL-7	Hs.72927	0.5	Mm.3825	26.1
IFNRB1			Mm.168	21.1
IFN a10			Mm.56966	20.4
VEGF-C	Hs.79141	-1.1	Mm.1402	16.9
VEGF-B	Hs.78781	-0.5	Mm.15607	16.2

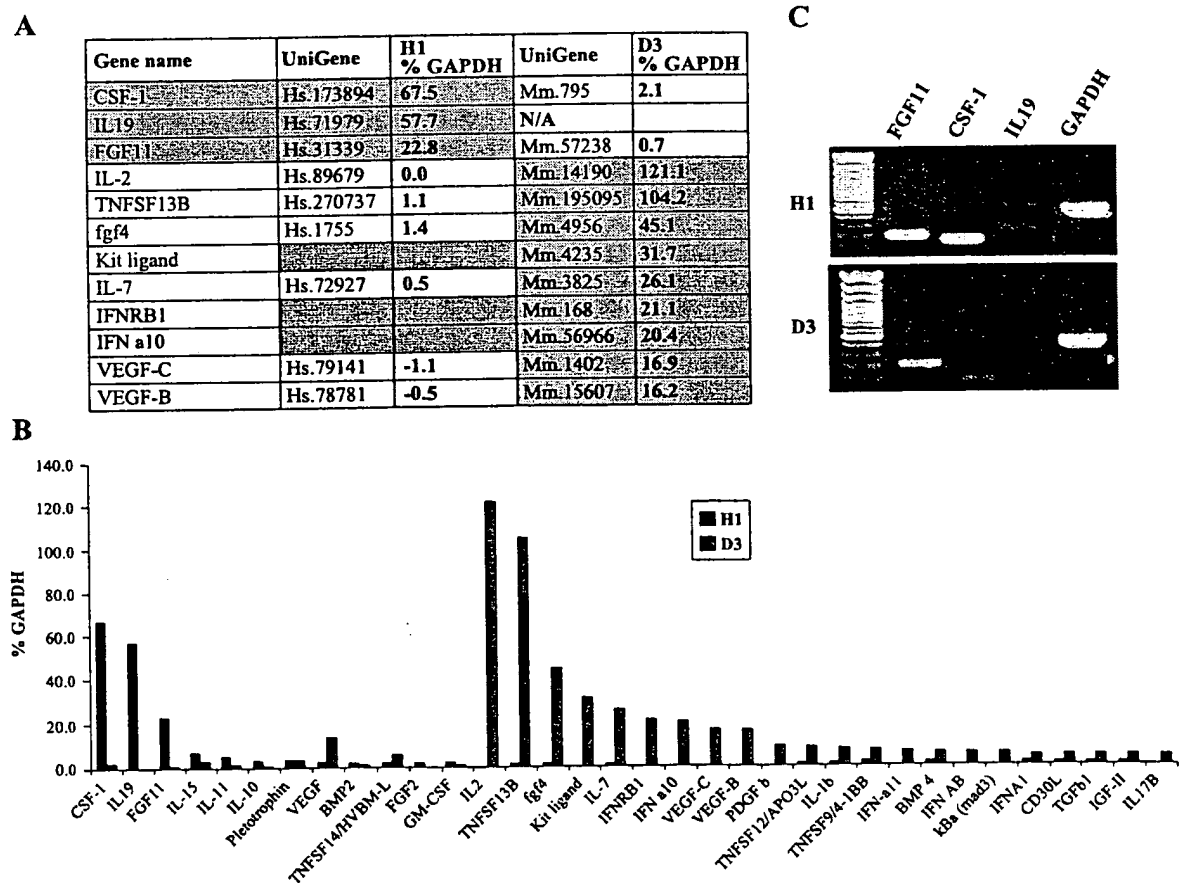


Fig. 6. Comparison of cytokine expression in undifferentiated mouse and human ES cells using cDNA microarrays. (A) The most prominently expressed genes in human and mouse ES cells: yellow—high expression, no fill—low expression; blue—no counterpart gene present on the array. (B) Quantification of the gene expression with ScionImage software. Each bar represents a mean of two separate experiments for each cell type. Only genes with expression of >4% GAPDH are presented. (C) RT-PCR results for genes highly expressed in human cells.

ly), a Src family protein tyrosine kinase, which participates in NF-kappa-B activation during B cell development. Many of the genes known to play an important role in cell death such as caspase 4/5, 8/9, FAS and FAS ligand were not expressed at detectable levels in either cell type. Human ES cells expressed, however, caspase 14, which was absent in mouse cells, while death receptor genes DR3 (UniGene: Hs.180338 and Mm.101198; 0% and 6.2%) and DR6 (which was only present on a mouse array, UniGene Mm.22649; 28.9%) were significantly expressed.

Overall these results show that both mouse and human cells are actively dividing cells, with mouse cells less protected from apoptosis consistent with the observation that many apoptotic cells are found in mouse ES colonies (see Fig. 1A). The pathways used to regulate cell cycle and apoptosis differ significantly with a limited overlap of genes.

Cytokine expression by ES cells

Given the difference between mouse and human marker expression and the differences in expression of cell cycle and cell death pathways genes, we felt that differences in cytokines may also be apparent. While cytokines can vary greatly and message levels tend to be low, the overall pattern of expression can nevertheless provide a basis for comparison. The cytokine microarray contained probes for major cytokine subsets: bone morphogenetic proteins (BMPs), colony-stimulating factors (CSFs), fibroblast growth factors (FGFs), interferons (INFs), insulin-like growth factors (IGFs), interleukines (ILs), platelet-derived growth factors (PDGFs), transforming growth factors (TGFs), tumor necrosis factors (TNFs), vascular endothelial growth factors (VEGFs), many of which are known to be active during development. As can be seen in Fig. 6, the overall patterns of cytokine expression in human and mouse ES cells were different (Fig. 6A). Expression of many of the genes shown to be elevated by microarray analysis was confirmed by EST scan (Brandenberger et al., submitted for publication) or MPSS (our unpublished results) and a smaller number were confirmed by RT-PCR (Fig. 6C). Few genes, IL-15, (Hs.168132), IL-11 (Hs.1721); pleiotrophin (Hs.44), VEGF (Hs.73793), BMP2 (Hs.73853), and TNFSF14 (LIGHT) (Hs.129708) were moderately expressed in both types of cells (Fig. 6B). The repertoire of cytokines expressed in mouse D3 cells was richer than in human cells (Fig. 6B).

LIFR/gp130 signaling pathways

We and others have noted that LIF is not required to maintain self-renewal of human ES cells, while it is critical for the maintenance of mouse cells. To identify the basis for the absence of the LIF requirement, we examined expression of both subunits of the LIF receptor: specific LIF receptor (LIFR) subunit and signal transducer gp130. As can be seen in Fig. 7C, LIFR message was absent in H1 cells, while LIFR could be readily detected in mouse

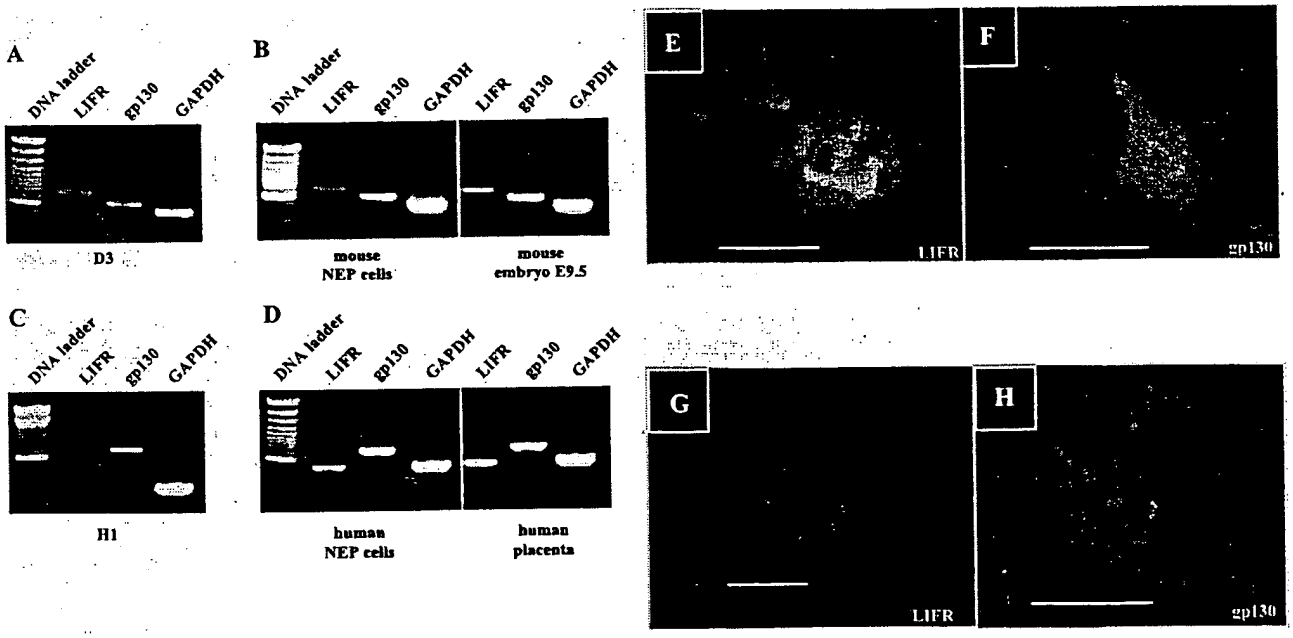


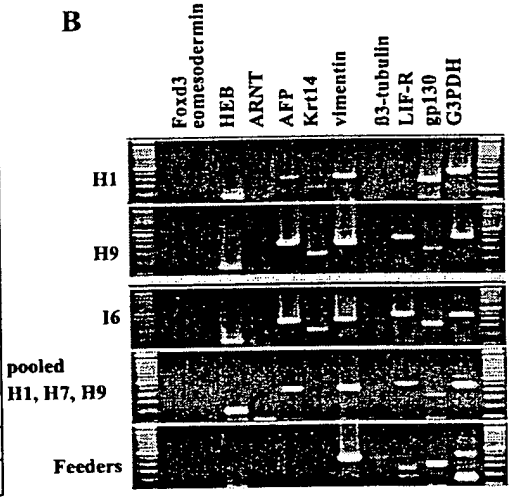
Fig. 7. Analysis of LIF receptor (LIFR) expression. (A–D) RT-PCR analysis. Expression of LIFR and its signal transducer gp130 in mouse D3 (A) and in human H1 cells (C). The same mouse and human primers and PCR conditions were used with control RNA samples from mouse neuroepithelial (NEP) cells and mouse E 9.5 embryo (B) and human NEP and human placenta (D). (E–H) immunostaining results. Mouse D3 and human H1 cells were stained with the antibody against LIFR (E and G, respectively) and with the antibody against gp130 (F and H, respectively). See Table 2 for antibody specifications.

ES cell cultures (Figs. 7A and B). H9 cells and pooled RNA preparations from H1, H7, and H9 cells had low levels of LIFR mRNA (EST analysis of these cells confirmed low expression of LIFR [1:1]), while the newly developed human ES cell line I-6 expressed significant levels of LIFR (see Fig. 8B). The failure to detect expression of LIFR in H1 cells could not be attributed to technical issues as it was verified with two different sets of LIFR primers (data not shown). Both sets of primers identified LIFR in human neuroepithelial cells and in human placenta (Fig. 7D). Absence of LIFR message in H1 cells was consistent with immunostaining results: no LIF receptor was detected on the surface of H1 cells (Fig. 7G), although the same antibody detected LIFR on mouse cells (Fig. 7E). Expression of gp130, a signal-transducing subunit of LIFR, was high in mouse cells and was readily detected by RT-PCR (Fig. 7A) and immunostaining (Fig. 7F). However, gp130 expression varied among human ES lines. H1 cells expressed low levels of gp130 gene (Fig. 7C), while no message for gp130 was found in H9 cells and in cells grown without feeders on Matrigel (H1, H7 and H9 RNA pool) (see Fig. 8B). The latter result is in accordance with zero EST counts for gp130 [0:6] in these cells. Since feeder cells also expressed gp130, it might be that the message for gp130 detected in RNA preparations from H1 cells can be attributed to contamination with feeder RNA. Indeed, immunofluorescence experiments showed quite low gp130 protein on the surface of H1 cells (Fig. 7H). The variable expression of gp130 and LIFR suggested that this pathway is not active at early stages of huES cell self-renewal and is consistent with our observations that several inhibitors of this pathway are actively transcribed (Brandenberger et al., Geron personal communication see also Table 5).

A

GENES	D3	H1
pluripotency		
FOXD3	yes	no
trophoblast		
eomesodermin	no	yes
HEB	no	yes
ARNT	yes	no
differentiation		
alpha fetoprotein	no	yes
keratin-14	no	yes
vimentin	no	yes
beta-III-tubulin	yes	no
LIF receptor	yes	no

B



C

array	genes expressed in H1 and D3	
	total	different
cell cycle	48	26
apoptosis	21	13
cytokine	30	25

D

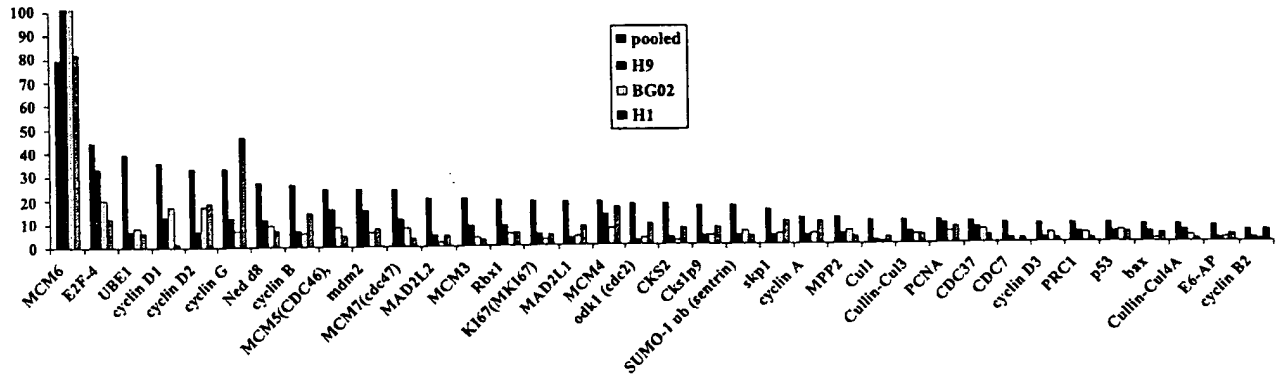


Fig. 8. Comparison of gene expression profiles in different human ES lines. (A) the list of genes that are differentially expressed in human H1 and mouse D3 ES cells according to RT-PCR analysis (see Figs. 3B–D and Table 3). (B) expression of these genes was analyzed by RT-PCR in RNA samples from H1, H9, I6 ES cell lines and from the pooled RNA sample of H1, H7, and H9 cells grown without feeders ("pooled") and from fibroblast feeder cells treated with mytomycin C and incubated with ES cell medium for 5 days ("feeders"). (C) summary of the differences between human H1 and mouse D3 cells in gene expression according to cDNA microarrays. (D) comparison of cell cycle gene expression in H1, H9, BN02 and "pooled" RNA samples using cell cycle cDNA microarray. Each bar is percentage of GAPDH.

Table 5
EST scan and MPSS were used to examine the expression of LIF pathway genes as described in Brandenberger et al. (personal communication)

Locusid	LIF pathway gene	MPSS tmp	EST scan
3589	Interleukin 11	nd	0
3976	ILIF	nd	2
3569	IL-6	nd	nd
5008	Oncostatin M	nd	nd
1489	Cardiotrophin 1	nd	nd
1270	CNTF	nd	nd
3977	LIFR	nd	1
5781	PTP, nonreceptor type 11	37	4
3572	gp130//IL-6RST	nd	0
3570	IL-6R	nd	nd
1271	CNTFR	nd	0
9180	Oncostatin M Receptor	nd	2
3718	Janus kinase 3	nd	0
3716	Janus kinase 1	22	0
3717	Janus kinase 2	nd	nd
9655	Suppressor of cytokine signaling 5	8	1
9306	Suppressor of cytokine signaling 4	nd	2
8651	Suppressor of cytokine signaling 1	nd	nd
122809	Suppressor of cytokine signaling 7	nd	1
9021	Suppressor of cytokine signaling 3	nd	0
51588	Protein inhibitor of activated STAT protein PIASy	107	5
8554	Protein inhibitor of activated STAT, 1	24	1
10401	Protein inhibitor of activated STAT3	54	5
6778	Signal transducer and activator of transcription 6	12	1
6774	Signal transducer and activator of transcription 3	nd	9
6772	Signal transducer and activator of transcription 1	55	4
6773	Signal transducer and activator of transcription 2	nd	0
6775	Signal transducer and activator of transcription 4	nd	nd
6776	Signal transducer and activator of transcription 5A	37	2
55250	Signal transducer and activator of transcription 3 interacting protein 1	34	2

Note the absence or low levels of expression of receptor and transducer components and the relatively high levels of inhibitors of the pathway such as phosphatase, PIAS and SOCS genes. nd = not detected, 0 = absent in ES cells but present after differentiation, tpm = transcripts per million, MPSS = massively parallel signature sequencing.

Differences between mouse and human ES cells are conserved in independently isolated ES cell lines

The significant differences observed between mouse and human ES cells in expression of pluripotency/differentiation markers, LIFR/gp130, apoptosis and cell cycle-related genes, and cytokines raised the possibility that differences were due to intrinsic differences in human and mouse cultures. Alternatively, these differences could arise from small differences in how cell lines were derived and maintained. To directly address this, we obtained RNA from two additional human ES cell lines: H9.2 clone of H9 and a newly derived line I-6 (Amit and Itskovitz-Eldor, 2002) that have been derived and propagated in a different lab. In addition, we tested pooled RNA sample from three different cell lines (H1, H9, and H7) that were propagated on Matrigel without feeders for 50 passages. Fig. 8A presents a list of markers that have been found differentially expressed in H1 and D3 cells (see Figs. 3B, C and D and Table 3). FOXD3, ARNT, beta-tubulin class III and GATA2 were not expressed in H1 cells and were present in D3 cells. As shown in Fig. 8B, all these genes were also absent in all human cell lines tested, while eomesodermin, HEB, AFP, and POTE (genes that were expressed in H1 but not in D3 cells) were present in other human cell lines as well. Keratin-14 message was absent in the pooled RNA from feeder-free human ES cells, and was present in feeder cell RNA, which confirms its origin from mouse fibroblasts.

Since microarray analysis provided the broadest overview of the cell's gene expression (see summary in Fig. 8C), we sought to investigate whether gene expression profile of H1 cells will be reproducible in other human ES cell lines. We performed cell cycle microarray analysis with H9 cells, with the human ES line BG02 from Bresagen and with pooled human ES cell RNA and with two additional lines (Fig. 8 and data not shown). A remarkable similarity in the pattern of expression of cell cycle genes among different populations of human ES cells was observed and this pattern was clearly distinct from that seen in mouse ES cells (Fig. 8D). Thus, it appears likely that the differences we observe between mouse and human ES cells reflect underlying fundamental differences in cell biology of the two populations.

Discussion

We have compared the expression of almost 400 genes in human and mouse ES cells that have been maintained in an undifferentiated state on MEF's under standard protocols. Our results show that mouse and human cells share similarities in expressing markers of the pluripotent state. The expression of these markers together with the absence of markers of differentiation constitutes a signature profile of undifferentiated ES cell cultures (Figs. 1–3, Table 3) and is similar to recent results reported by microarray comparison (Sato et al., 2003). The immunostaining markers and validated primers reported in this study will form the basis for developing a well-tested set of markers, which will allow careful, regular monitoring of ES cells in culture. It is important to note that the primers used in this study have been verified to amplify specific cDNA fragments in control

mouse and human fetal tissues using identical RT-PCR protocols. Band sizes were selected such that multiplex RT-PCR could be readily performed or amplified DNA fragments could be used to develop focused ES cell microarrays. However, signature profiles described in mouse and human ES cells were not identical. Apart from previously reported differences in expression of embryonic antigens (Henderson et al., 2002) and the ability to differentiate into trophoectoderm (Odorico et al., 2001; Thomson et al., 1998a), other differences between human and mouse ES cells have been identified in these studies. These differences were conserved in all human ES lines tested (Fig. 8 and Table 6).

An important difference that we have noted is LIF receptor expression. LIFR message was low or absent in H1 and variable in all human ES lines tested, while LIFR could be readily detected in mouse ES cell cultures. Our failure to detect LIFR and gp130 was confirmed by an EST scan where we likewise detected inconstant/low levels of LIF receptor (Brandenberger et al., submitted for publication; Carpenter et al., 2004; Rosler et al., 2004). These results are consistent with the lack of a LIF requirement for maintenance of undifferentiated human ES cell lines and with the altered LIF signaling observed in human teratocarcinoma lines (Schuringa et al., 2002) and the high levels of suppressors of the cytokine signaling pathway (Brandenberger et al., submitted for publication). Interestingly, EST enumeration detected significant levels of LIF embryoid bodies [2:9] suggesting that LIF may play a role in differentiation of these cells. Elucidation of the role of LIF in paracrine regulation of ES cells requires further investigation.

The presence of a LIF-independent self-renewal pathway has been described in mice (Dani et al., 1998) though it is thought to play a minor role in self-renewal (reviewed in Burdon et al., 2002; Niwa, 2001). It is possible, however, that this pathway plays a larger role in human ES cell cultures. More recently, a homeobox protein, nanog, was shown to be involved in self-renewal via activation of a LIF independent pathway (Chambers et al., 2003; Mitsui et al., 2003). Nanog expression is conserved in all human lines examined so far (Brandenberger et al., submitted for publication).

Table 6
Distinguishing features of mouse and human ES cells

Marker	Mouse	Human
Morphology	More diverse	Rounded with sharp boundaries
SSEA-1	Present	Absent
SSEA-4	Absent	Present
Vimentin	Absent	Present
Trophoectoderm markers	Absent	Present
β-III tubulin	Present	β-5 tubulin present
LIFR	High	Low/variable
Gp130	High	Low/variable
FGF4	High	Absent
HRASP	Required	Pseudogene
E-hox	Required	No orthologue present
Fox-D3	Present/required	Low/absent

Major differences between mouse and human ES cells (see also Fig. 8) based on present results and unpublished results (Brandenberger et al., personal communication) are summarized.

Another surprising difference between human and mouse ES cells, which we have noted, is absence of FoxD3 expression. The results of EST scan for FoxD3 [0:0] were consistent with our RT-PCR results although it should be noted that some reports (reviewed in Carpenter et al., 2003) have suggested expression of FoxD3 in human ES cells. In contrast to human cells, FoxD3 was readily detected in mouse ES cell cultures. FoxD3 or GENESIS is expressed early in mouse embryonic development, and abrogation of FoxD3 function leads to a failure of the blastocyst to develop beyond the four-cell stage (Hanna et al., 2002). In addition, FoxD3 is critical for endodermal differentiation when it antagonizes the activity of OCT3/4 (Guo et al., 2002). Differences in FoxD3 expression between mouse and human ES cells, if verified, would further illustrate fundamental differences in the biology of these cells.

Our studies of patterns of expression of cell cycle, apoptosis, and cytokine genes uncovered additional fundamental species-specific differences. While it is impossible to discuss all findings, it is worth highlighting a few critical differences that are not only of quantitative nature, but suggest that different signaling pathways are operative and thus may provide some insights on ES cell biology. For instance, components of a p53 pathway that controls growth arrest and cell death in response to DNA damage (Gottifredi et al., 2000; Schultz et al., 2000) were much more active in mouse ES cells, while human ES cells expressed TNF receptor 2, which has been implicated in a survival signaling cascade (Peschon et al., 1998) probably through NF-kB activation (Dopp et al., 2002). Even when similar pathways were utilized, different members of the same family were more or less abundant. MCM6, for example, was high in human cells and MCM-2, MCM-3, and MCM-7 were high in mouse cells. Overall, the differences observed in array analysis between human and mouse ES cells were unexpectedly large, which suggests that caution needs to be exercised in extrapolating from studies in mouse cells.

Our analysis identified other unexpected findings. Human ES cells expressed additional markers of trophoectoderm, eomesodermin (Russ et al., 2000), and HEB (Janatpour et al., 1999), which was consistent with observations that human ES cell lines could differentiate into trophoectoderm (Odorico et al., 2001; Thomson et al., 1998a). An additional surprising observation was the presence of a contaminating population of vimentin-immunoreactive cells interspersed within undifferentiated ES cells. Message for vimentin was present in RNA preparations as well. Vimentin-positive cells appear intercalated among the other cells in the colonies and cannot be readily distinguished by morphology. However, double immunostaining

with the marker of undifferentiated cells TRA-1-81 showed no co-localization, suggesting that these cells are differentiated cells. Mouse cells did not express vimentin but expressed beta-tubulin class III, which co-localized with SSEA-1. Recently, ES cells were found to express beta-tubulin class V (unpublished results), which might cross-react with our reagents.

Two alternative possibilities could explain numerous differences observed between mouse and human ES cells. One possibility is that culture conditions for growing human ES cells were not optimized for maintaining the cells in an undifferentiated state, and that the differences that we observed will be resolved as culture conditions improve. The observed differences may reflect some degree of differentiation, atypical expression of certain markers or transdifferentiation. We note, for example, the small number of differentiated cells of unknown character that were present in human ES cell cultures, and the expression of several markers of differentiated cells such as vimentin, keratin14, and beta III tubulin. However, our initial examination of additional human ES cell lines suggests that culture conditions alone could not explain the observed differences between mouse and human ES cells. Besides the H1 line, we have studied H9 cells and the newly developed line I-6, as well as ES cells grown without feeders for 50 passages and cell lines from Bresagan. The gene expression patterns in all these lines appeared similar to each other and differed from mouse ES cells. Since these lines were grown in different laboratories under distinct culture conditions, we would argue that observed dramatic differences between human and mouse cells reflect underlying differences in the biology of the cells rather than differences secondary to a response to the culture environment. However, we suggest that a careful assessment of a larger number of lines with additional markers will be required to make a final determination.

An alternate, equally plausible, explanation for the differences between mouse and human cells is that human ES cells may have been isolated at a slightly different perhaps earlier stage of blastocyst cell maturation, and thus the observed differences reflect the stage of harvesting of ICM. This possibility is consistent with the reported ability of human ES cells (and the inability of mouse ES cells) to differentiate into trophoblast or to contribute to placental development in vivo, and with our current observations of the expression of several early trophoectoderm markers and the expression of some of the putative differentiation markers such as AFP and GATA4 that would be consistent with the possibility that early harvested ES cells may retain the capability to make extra-embryonic endoderm and mesoderm.

In summary, our results show that the overall strategies to regulate self-renewal and differentiation are likely similar among ES cell lines from different species. However, significant differences are present and it will be important to identify and characterize such differences before a useful set of markers for the differentiated and undifferentiated state can be developed. Once such a marker set is developed and validated, it is likely that it can be used across all the 60 or so lines that have been identified. Our future experiments will be directed at extending the analysis to other lines as they become available and understanding the functional significance of the molecular differences we have observed.

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Signalling through the JAK–STAT pathway in the developing brain

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The JAK–STAT (Janus kinase–signal transducer and activator of transcription) signalling pathway that is stimulated by cytokines has been much investigated in haematopoietic cells, but recent data indicate that this pathway is also present and active during neuronal and glial differentiation. Furthermore, it is now clear that growth factors other than the classical cytokines can act through this pathway and that physiological inhibitors of this signalling cascade exist. Thus, the JAKs, the STATs and their specific inhibitors could be molecules with important roles in the CNS.

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CELLULAR DIVERSITY in the CNS evolves from the concerted processes of cell proliferation, differentiation, migration and survival, and synapse formation. There is now strong evidence to indicate that most of these events are regulated by growth factors and cytokines. Two major classes of cell-surface receptors for these factors have been identified: (1) receptors that display intrinsic tyrosine-kinase activity, also known as receptor-protein tyrosine kinases (RPTKs), that mainly signal through the Shc–Grb2–Ras–MAPK (mitogen-activated protein kinase) pathway[1]; and (2) receptors such as the cytokine receptors, which lack intrinsic catalytic kinase domains and function through cytoplasmic kinases[2]. While the former have been the subject of recent reviews, cytokine receptors and, in particular, cytokine signalling, represent a more-recent area of CNS research. This article focuses on the most well-described cytokine signalling proteins, and reviews the evidence that they are both present and active during neuronal and glial differentiation.

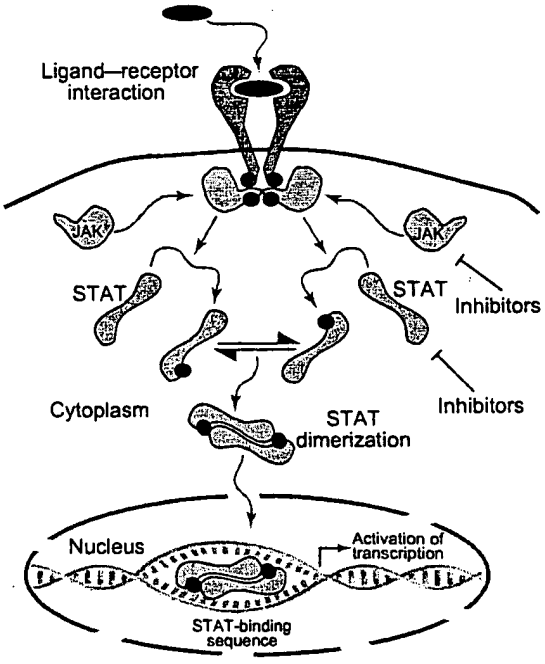

Fig. 1. *Signalling through the JAK–STAT pathway. The interaction of a cytokine (black) with its specific receptor (dark green) induces receptor-complex dimerization. This brings members of the JAK cytoplasmic kinases (yellow) into juxtaposition, allowing them to transphosphorylate each other and to phosphorylate specific STAT proteins (pale green). STATs are found in the cytoplasm in monomeric form and, following tyrosine-residue phosphorylation by the activated JAKs, they associate with the cytokine receptor, homo- or hetero-dimerize, translocate to the nucleus and bind to specific DNA elements (STAT-recognition sites) that are situated upstream of the genes whose expression is induced by cytokines. Abbreviations: JAK, Janus kinase; STAT, signal transducer and activator of transcription.*

Cytokine signalling: from membrane to nucleus

Signalling from cytokine receptors has been thoroughly investigated in the haematopoietic system, where it has been found to involve two superfamilies of cytoplasmic signalling proteins, the JAKs (Janus kinases) and the STATs (signal transducer and activator of transcription) (Fig. 1)[2–5]. On activation of a cytokine receptor, one or more of the four known JAKs (JAK1, JAK2, JAK3 and TYK2) are recruited to the membrane (Figs 1 and 2). Subsequently, the activated JAKs phosphorylate the tyrosine residues of STATs, which stimulates them to translocate to the nucleus. Once in the nucleus, phosphorylated STATs interact with specific DNA elements (the STAT recognition sites) situated upstream of genes whose expression is induced by cytokines (Fig. 1). Seven members of the STAT family have been described in mammals to date, STAT1, STAT2, STAT3, STAT4, STAT5a and STAT5b, and STAT6 (Fig. 2). Most cytokine receptors employ different combinations of JAKs and STATs in order to control the specificity of the ensuing transcriptional events[2–5] (for molecular and biochemical details of the JAK–STAT pathway see Refs 2–5).

Activation of STATs can also occur independently of JAK activation and in response to stimuli other than the classical cytokines. For example, STAT1 and STAT3 are phosphorylated by activated RPTKs, such as epidermal growth-factor and fibroblast growth-factor receptors[6,7]. In view of these observations, and the discovery that cytokine receptors, RPTKs and JAKs–STATs are distributed throughout the developing brain[8–12], it is conceivable that these signalling proteins are involved in a diverse array of activities in the CNS, some of which have yet to be described.

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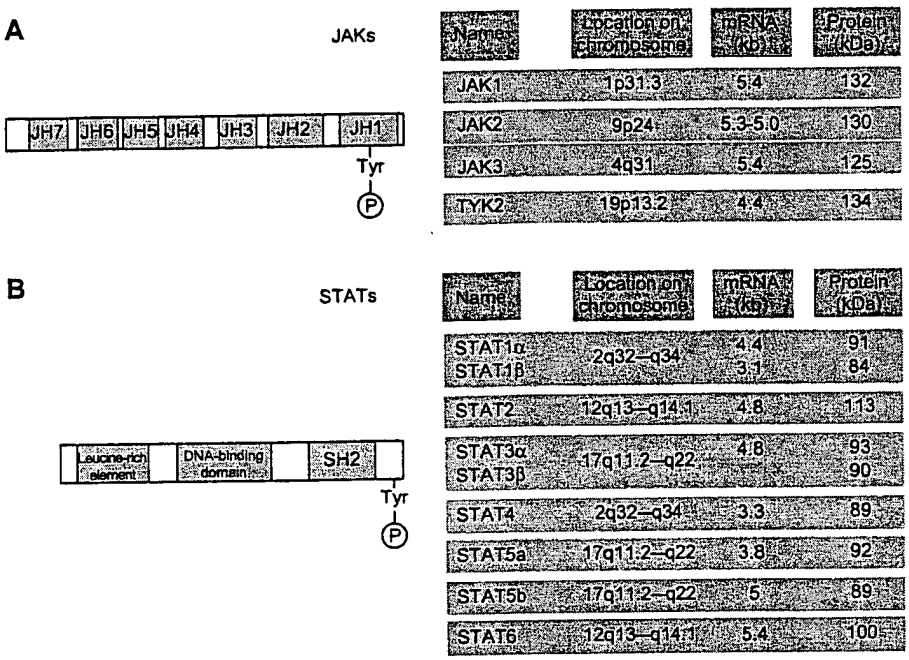

Fig. 2. *The JAK and STAT families of intracellular signalling proteins.* **(A)** *The four known JAK proteins are characterized by seven JH domains. The JH1 domain that contains the tyrosine-kinase and phospho-tyrosine sites is indicated.* **(B)** *The seven members of the STAT family share conserved structural and functional domains: (1) a conserved SH2 (SRC-homologous 2) phosphotyrosine-binding domain; (2) the phosphotyrosine at the C terminus; (3) a DNA-binding domain; (4) a leucine-rich 'hapted repeat' element that is important for the regulation of the phosphorylation process; and (5) a functional serine phosphorylation site (identified on STAT1 and STAT3; see Refs 2–5 and references therein). Abbreviations: JAK, Janus kinase; STAT, signal transducer and activator of transcription.*

The JAKs–STATs enter the brain

The existence of JAK-family members in the nervous system was first reported by Yang *et al.*, who provided evidence of mRNA for JAK1 in the retina and in whole-brain extracts during development[13]. Subsequent immunohistochemical analyses have shown that dividing and postmitotic cells in the rat brain all exhibit immunoreactivity for anti-JAK2 antiserum[12] at embryonic day 16 (E16). Elevated JAK2 levels in various brain regions during embryonic and postnatal stages were confirmed by western-blot analysis of lysates[12]. Very low levels of JAK3 were found, while TYK2 (tyrosine kinase 2) was not detected[12].

Of more immediate relevance is the analysis of the STATs, as these are directly involved in gene activation. An *in vivo* ontogenetic study of the rat brain revealed a marked specificity in the pattern of expression of the genes encoding particular STAT-family members[10,12]. Western-blot analysis showed that STAT6 protein was abundant in various brain regions (hippocampus, striatum, cortex and basal forebrain) between E14 and E18, but progressively declined in lysates from more-mature stages of development (postnatal days 0, 2, 10 and adult) (Fig. 3A). Progressively reduced levels of STAT6 protein were also observed in primary striatal cells that were induced to differentiate into neurones in serum-free media[12] (Fig. 3B), a result that is consistent with the *in vivo* data.

A limited number of ligands have been reported to activate STAT6 in non-neuroneal cells, namely interleukin 4 (IL4), IL13 (whose receptor shares its α subunit with the IL4 receptor), IL3 and platelet-derived growth factor (PDGF)[14–16]. Investigation of the presence of these factors and their receptors, and the expression of the genes that encode them during brain development has revealed overlapping profiles for the PDGF receptor (PDGFR, both the α and β subclasses) and STAT6 (Refs 12,17, 18). Thus, strong immunoreactivity and high levels of mRNA were observed for the PDGFR in the forebrain of E11 mice (corresponding to E13 in the rat), these peaked at E16 and then declined to almost undetectable levels in the adult brain[17,18]. Platelet-derived growth factor is implicated in gliogenesis in the CNS (Ref. 19), and recent studies have demonstrated that it has a role in the neuronal differentiation of ventricular-zone progenitor cells in the cortex of E14 rats[20]. The various forms of this ligand, PDGFAA, PDGFBB and PDGFAB, are also present during development *in vivo*[18,21,22]. Taken together, these observations provide good circumstantial evidence to indicate that PDGF is a potential activator of STAT6 in the CNS. Other molecules besides PDGF, such as IL3, a differentiation factor for septal neurones (reviewed in Ref. 23), or other undescribed ligands, might also transduce through STAT6 in the brain.

Recent *in vitro* data have shown that STAT5, STAT3 and STAT1 are involved in the division, survival and differentiation of embryonic CNS cells[24–29]. Consistent evidence of their presence *in vivo* has also been reported[12]. As observed with STAT6, the levels of STAT5 and STAT1 also change in the various brain regions during development[12] (Fig. 3A), suggesting that there is active regulation of the availability of these transcription factors during development.

In an attempt to identify functional roles for STATs in the brain, and prompted by the fact that STAT5 is found in the embryonic striatum[12] (Fig. 3A), the activation of this transcription factor and whether it is of biological significance for striatal progenitor cells was investigated. Together with JAK2, STAT5 is a component of the IL3 signalling pathway[30]. In order to activate this pathway the gene encoding the IL3 receptor was overexpressed in striatal-derived ST14A progenitor cells[31]. The addition of IL3 to serum-deprived cultures resulted in an increased phosphorylation of STAT5 (and JAK2), and subsequent proliferation of the cells[26]. These data could, thus, indicate that there is a link between activated STAT5 and mitogenesis in the embryonic brain.

Planas *et al.*[11] reported constitutive *Stat3* expression and activation in various cell types of the rat cerebellum during postnatal development and adulthood, including granular and Purkinje neurones and glial cells. Analysis of STAT3 levels in the striatum, hippocampus, cortex and basal forebrain revealed similarly invariant protein levels[12] (Fig. 3A), indicating that it could have important roles in the different brain regions at various stages of brain maturation. As discussed

in greater detail below, phosphorylated STAT3 (and STAT1) are implicated in the differentiation of specific CNS cell types[24,25].

Despite the available data on the existence (and activation) of the JAKs–STATs in CNS cells, no major brain defects have been identified in animals where the JAKs or STATs were knocked out[32–39]. The evidence discussed in this article indicates, however, that this could simply reflect the existence of functional redundancy among members of the same family of proteins. In the case of *Jak2* and *Stat3* knockouts[40–42], the early embryonic lethality observed in these mice could be caused by the absence of these proteins during the primary stages of development of the nervous system, as discussed in this article.

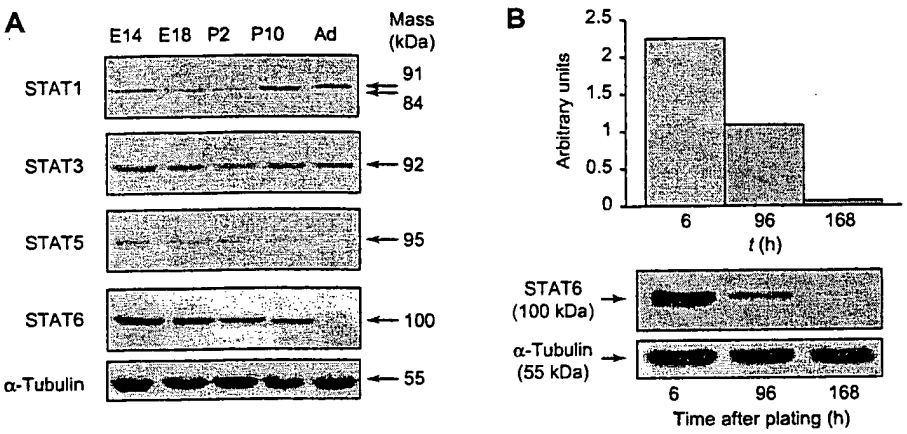

Fig. 3. Protein levels of STAT1, STAT3, STAT5 and STAT6 during development in the rat striatum. (A) Western-blot analysis of rat striata at various stages of development, from embryonic-day 14 to postnatal-day 10 (E14–P10) and in adults (Ad). A similar pattern was observed in all other brain regions[12]. The arrows indicate the appropriate mass of each STAT. STAT4 was not detected; species-specific antibodies for STAT2 were not available. (B) Western-blot analysis of STAT6 levels in undifferentiated (6 h) and differentiated (96 h and 168 h) primary neuronal cultures prepared from E14 rat striatum. Normalization of STAT6 levels with respect to α-tubulin content is reported in the graph. Abbreviation: STAT, signal transducer and activator of transcription. Reproduced, with permission, from Ref. 12.

Phospho-STAT-dependent differentiation by ciliary neurotrophic factor (CNTF) and CNTF-like ligands: interference by MAPKs?

Most of our current knowledge on the role of the JAK–STAT pathway in the CNS derives from studies that have used CNTF (Ref. 43) as the triggering factor. Among its various effects, CNTF is a known inducer of astroglial differentiation from both the O2A progenitor[44,45] and the CNS precursor cells[24,25]. Molecular dissection of this event has revealed that CNTF-triggered differentiation of immature CNS progenitor cells into glial fibrillary acidic protein (GFAP)-producing cells requires the phosphorylation of tyrosine residues in JAK1, STAT1 and STAT3 (Refs 24,25). In non-neuronal cells, phosphorylation of STAT1 (and STAT3) is correlated with increased transcription of *Cdkn1a* (a cell-cycle blocker), also known as *Waf1*, and suppression of cell division[6], and also with the generation of an anti-apoptotic signal, most probably through the induction of the *Bcl2* or *Bclx* genes (see Refs 5,46). Although not yet demonstrated in CNS cells, similar downstream activities of phosphorylated STAT1 and STAT3 might be necessary for proper glial-cell differentiation in the brain.

Examination of the presence of putative STAT-recognition sites within the GFAP promoter has revealed the existence of seven *cis*-elements to which the activated STATs bind[28]. Deletion of these elements or expression of the dominant negative STAT3 and STAT1 prevents GFAP synthesis[24,28]. These studies are suggestive of a correlation between CNTF, the JAKs–STATs and aspects of glial-cell differentiation.

Within this same differentiation paradigm, the role of the MAPK pathway is less clear, although it is also activated by CNTF. One study has reported that inhibitors of the MAPK pathway augment CNTF induction of the GFAP promoter[24], suggesting that JAK–STAT activation favours GFAP expression, while phosphorylated MAPKs have a negative effect on this event. In a second study[25], however, activation of both MAPKs and JAK–STAT pathways were found to be positively coupled to astrocytic differentiation *in vitro*. In this study, exposure of the cultures to PD098059, the inhibitor of MAPKK (MAPK-kinase, the upstream activator of MAPK) was shown to cause a delay in astrocytic differentiation. Therefore, it has been concluded that the two pathways are activated with different kinetics to control early (involving the MAPKs pathway) and late (involving the JAK–STAT pathway) phases of glial-cell differentiation[25]. Differences in the culture conditions and also in the doses of CNTF used in the two studies might account for the differences observed: indeed, recent evidence from Monville *et al.* indicates that different doses of CNTF might elicit or inhibit GFAP synthesis, depending on quantitative differences in recruitment of the members of these two pathways[47].

A function for the MAPK pathway, in conjunction with the JAK–STAT pathway, has also been revealed from analysis of a different cellular system, the O2A progenitors of the optic nerve[48,49]. In this system it was found that cytokine signalling, by CNTF, IL6 or leukemia inhibitory factor (LIF) together with tyrosine-kinase signalling (from, for example, insulin-like growth factors or neurotrophin 3) are both necessary for proper development and survival of oligodendrocytes. Indeed, factors that act within the same class of receptors (cytokine receptors or RPTKs) only promote short-term survival of the same cells[48,49].

The existence of crosstalk between the JAK–STAT and Ras–MAPK pathways has also been demonstrated in PC12 cells exposed to IL6 using the classical neurite outgrowth assay (see Ref. 5 for a review). Wu and Bradshaw[50,51] and Ihara *et al.*[52] found that IL6-induced phosphorylation of STAT3 could be a negative regulator of MAPK-dependent neurite extension. A complex interplay between activating growth factors and signalling pathways might thus provide a strategy for proper temporal control of the genesis, and quantitative and qualitative characteristics, of mature brain cells.

Finally, it is interesting to note that, in contrast to its role in glial-cell differentiation, phosphorylated STAT3 acts to suppress differentiation in LIF-stimulated

TABLE 1. Soluble factors demonstrated to act through the JAK–STAT pathway in the nervous system

Ligand	JAK	STAT	Cell system
IL3*	JAK2 (Ref. 26)	STAT5 (Ref. 26)	Striatal-derived ST14A cells[26]
IL6		STAT1, STAT3 (Refs 50–52)	PC12 cells[50–52] (reviewed in Ref. 5)
IL15	JAK1 (Ref. 55)		Microglial cells[55]
IFNγ	JAK1 (Ref. 56); JAK2 (Ref. 57)	STAT1 (Refs 56–58)	Cultured glial cells[56,57], primary septal cells[58]
CNTF	JAK1 (Ref. 24)	STAT1, STAT3 (Refs 24,25,27,59)	CNS progenitor cells[24,25] septal-derived immortalized cells, CNS neuronal cultures[27,59]
LIF		STAT1, STAT3 (Ref. 59)	Cultured sympathetic neurones[59]
OSM		STAT1 (Ref. 60)	Sympathoadrenal progenitor cell line[60]
SGDA		STAT3 (Ref. 61)	Sympathetic neurones[61]
Leptin		STAT3 (Ref. 62)	Hypothalamus (*in vivo*)[62]

*Exogenous IL3-receptor chains.

Abbreviations: CNTF, ciliary neurotrophic factor; IFNγ, interferon γ; IL, interleukin; LIF, leukemia inhibitory factor; JAK, Janus kinases; OSM, oncostatin M; SGDA, sweat-gland-derived differentiation activity; STAT, signal transducer and activator of transcription.

embryonic stem cells[53]. In this system, effect might occur via induction of cyclin-dependent-kinase activators[54]. Cell-type specific interpretation of STAT3 activation thus appears to be pivotal in the diverse developmental effects of the LIF–CNTF family of cytokines[53].

Besides the cytokines mentioned so far, other factors that produce their effects through the JAKs–STATs have been identified in neuronal cells (see Table 1). Similarly, together with *Gfap*, other genes with clear relevance to the CNS have been identified that contain a STAT-recognition site in their promoters (see Table 2).

TABLE 2. Genes of interest to the field of neurobiology that are targets (or potential targets) of phosphorylated STATs

Gene or promoter	STAT involved	Refs
Casp1, *Casp2*, *Casp3*	STAT1	63,64
Cdkn1a (also known as *Waf1*) promoter	STAT1, STAT3 and STAT5	6,65
Bclx promoter	STAT1	46, reviewed in 5
Vip promoter	STAT1 or STAT3	59
Chat promoter	not determined	59[a]
Tak1 promoter	not determined	59[a]
Cgrp promoter	not determined	59[a]
Penk promotor	not determined	59[a]
Cck promoter	not determined	59[a]
Junb promoter	STAT3	66
Fos promoter	STAT1 or STAT3	67
Icam1 promoter	STAT1 or STAT3	68[a],69[a]
Gfap promoter	STAT1 or STAT3	24,28
Psen1 promoter	STAT1 or STAT3	70[a]

For each gene, the table indicates the STAT involved in the induction of its expression.

[a]The genes in which a STAT consensus sequence has been identified through a database search: this analysis is not conclusive of an interaction between the STAT and the DNA element.

Proteins encoded by the genes shown: *Bclx*, the BCL family of proteins; *Casp*, caspases; *Cck*, cholecystokinin; *Cdkn1a*, cyclin-dependent kinase inhibitor 1A; *Cgrp*, calcitonin-gene-related peptide; *Chat*, choline acetyl transferase; *Fos*, FOS (formerly c-fos); *Gfap*, glial fibrillary acidic protein; *Icam1*, intercellular adhesion molecule 1; *Junb*, JUNB; *Penk*, enkephalin; *Psen1*, presenilin 1; *Tak1*, substance P (tachykinin 1); *Vip*, vasoactive intestinal polypeptide.

Abbreviation: STAT, signal transducer and activator of transcription.

New players: the physiological inhibitors of the JAK–STAT pathway

Two main classes of physiological inhibitors of the JAK–STAT pathway have recently been identified in haematopoietic cells. These are: (1) molecules such as suppressor of cytokine signalling 1 (SOCS-1), also known as JAB (JAK-binding protein) or SSI [STAT-induced STAT inhibitor 1 (Refs 71–73)], which are JAK-binding proteins that function by reducing the tyrosine-kinase activity of the JAKs, thereby suppressing the activation of the STATs; and (2) protein inhibitor of activated STAT (PIAS) molecules, such as PIAS3, that specifically binds to STAT3 and inhibit its DNA-binding activity and, therefore, its ability to activate genes[74]. Molecules that interfere with phosphorylated tyrosine residues on specific cytokine receptors have also been identified[75]. Interestingly, transcription of the genes for these JAK–STAT-pathway inhibitors is positively modulated by various cytokines, suggesting that they could function in a negative-feedback loop that regulates JAK–STAT signalling[71–75].

These molecules might also act in an analogous manner in the CNS and influence JAK–STAT signalling. Indeed, it has been suggested that the overall strength of STAT signalling for a given cell type could be largely affected by the relative level of expression of the genes encoding the STATs and their inhibitors[74]. The presence and role of these inhibitors in the brain await further analysis. Preliminary evidence indicates that SOCS-1 is present in both the developing and the mature brain (E. Cattaneo and C. De-Fraja, unpublished observations).

Concluding remarks

The evidence summarized in this article indicates that the JAK–STAT signalling proteins are present and activated in the brain, where they intervene physiologically in order to modulate aspects of brain maturation. As cells mature, changes in the levels and activity of these cytoplasmic signalling proteins occur that might influence the responsiveness of the cells to growth factors or cytokines. An analogous hypothesis has recently been proposed for SHC (SRC homology 2 domain-containing transforming protein C), a key

adaptor molecule that acts downstream from activated RPTKs[1,76].

It might also be speculated that the mechanisms by which cell proliferation, survival and differentiation occur during development are reactivated in adult cells after damage, and the variations in the synthesis or activity, or both, of the JAK–STAT-family member in those conditions might contribute to or attempt to counteract pathological conditions in the adult brain[77–79]. In neurodegenerative disorders, injured or dying neurones might well prepare the molecular machinery that increases or modifies their responsiveness to cytokines and growth factors, thereby influencing their survival or neurite elongation, or both[80]. In agreement with this possibility, selective and substantial upregulation of the expression of *Jak2* and *Jak3* (as well as of specific cytokine receptors) has been reported after peripheral injury[80]. In the same paradigm, interesting variations in the levels of SHC adaptors have also been observed[81] (see Ref. 1 for a review).

This article has focused on events that occur during CNS development in which the JAK–STAT proteins are involved. Additional functions for these proteins have been identified in the mature brain[62,82]. Despite the fact that they were initially identified in the haematopoietic system, the JAKs and the STATs have already made an important entry into neurobiology.

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The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium

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SUMMARY

The *in vitro* developmental potential of mouse blastocyst-derived embryonic stem cell lines has been investigated. From 3 to 8 days of suspension culture the cells form complex embryoid bodies with endoderm, basal lamina, mesoderm and ectoderm. Many are morphologically similar to embryos of the 6- to 8-day egg-cylinder stage. From 8 to 10 days of culture about half of the embryoid bodies expand into large cystic structures containing alphafoetoprotein and transferrin, thus being analagous to the visceral yolk sac of the postimplantation embryo. Approximately one third of the cystic embryoid bodies develop myocardium and when cultured in the presence of human cord serum, 30% develop blood islands, thereby exhibiting a high level of organized development at a very high frequency. Furthermore, most embryonic stem cell lines observed exhibit similar characteristics. The *in vitro* developmental potential of embryonic stem cell lines and the consistency with which the cells express this potential are presented as aspects which open up new approaches to the investigation of embryogenesis.

INTRODUCTION

Blastocyst-derived embryonic stem (ES) cells are established *in vitro* from substrate-attached blastocysts without passage of the cells through tumours. They are maintained in an undifferentiated pluripotent state by culturing on an embryonic fibroblast feeder layer and spontaneously differentiate in the absence of feeder layer cells. Several methods have been applied successfully to the establishment of ES cell populations. Evans & Kaufman (1981) produced lines of pluripotent cells from the epiblast of delayed-implantation blastocysts. At the same time Martin (1981) established cell lines from cultures of immunosurgically isolated inner cell mass cells which were grown in medium conditioned by teratocarcinoma-derived embryonal carcinoma (EC) cells. It is now known that ES cell lines can be established without delayed-implantation blastocysts or EC-cell-conditioned medium (Robertson, Evans & Kaufman, 1983; Axelrod, 1984). There is no indication that there are significant differences between the cell populations derived in these ways.

Key words: ES cells, visceral yolk sac, blood islands, myocardium, mouse embryo.

Teratocarcinoma-derived EC cells are produced from a limited number of mouse strains which have the capacity to develop teratocarcinomas and have been used as an *in vitro* model system for embryonic development (Solter & Damjanov, 1979; Martin, 1980). The *in vitro* differentiation potentiality of EC cells has been studied either with the help of chemical inducers (Jones-Villeneuve, McBurney, Rogers & Kalnins, 1982; McBurney, Jones-Villeneuve, Edwards & Anderson, 1982; Paulin *et al.* 1982) or with cell lines which spontaneously differentiate under varying culture conditions (Rosenthal, Wishnow & Sato, 1970; Martin & Evans, 1975*a, b*; Sherman & Miller, 1978; Darmon, Bottenstein & Sato, 1981; Pfeiffer *et al.* 1981; Rizzino, 1983). Few EC cell lines are capable of spontaneous differentiation, and of these very few have the capacity to form cystic structures with phenotypic similarities to the postimplantation embryo (Rosenthal *et al.* 1970; Martin, Wiley & Damjanov, 1977; Cudennec & Nicolas, 1977).

The cells of blastocyst-derived ES cell lines may be quite similar to normal embryonic cells and in most cases are probably less altered by their *in vitro* environment than are the cells of most teratocarcinoma-derived cell lines by a tumour environment. This is most clearly evidenced by the remarkably high frequency with which ES cells can be used in blastocyst injection experiments to form chimaeras of a broad tissue spectrum as well as germ-line chimaeras (Bradley, Evans, Kaufman & Robertson, 1984). Other advantages of ES cells lie in the fact that they can be made from mouse strains which carry recessive lethal mutations (Magnuson, Epstein, Silver & Martin, 1982) or from parthenogenic embryos (Robertson *et al.* 1983). The degree, however, to which the use of ES cell lines will be beneficial in investigating the lesions occurring in such strains will be largely dependent upon the degree to which the lesion-bearing lines and the non-lesion-bearing lines of the same genetic background will be able to provide some semblance of organized *in vitro* development. It is therefore necessary to know the developmental potential of these cells in order that the fullest possible range of questions can be directed within the boundaries of this potential.

All blastocyst-derived ES cell lines so far described spontaneously differentiate and form cystic embryoid bodies (Evans & Kaufman, 1981; Martin, 1981; Robertson *et al.* 1983). The degree to which organized development similar to that of the embryo occurs within them, however, has not been described. The investigation reported here has done this by analysing the most advanced embryonic-like structures developed by a blastocyst-derived cell line, ES-D3. It has compared the extent of this development, as well as that of several other ES cell lines from 129 and C57 mouse strains, to the postimplantation embryo. It is shown that the blastocyst-derived cells can differentiate at a remarkably high frequency to form heart and blood cell-containing cystic structures similar to the visceral yolk sac of the embryo. A close analysis is made of the fluid content of the cystic structures, the erythrocytes of the blood islands and the morphology of the heart-like structures. The unique advantages which ES cells may provide to the study of embryonic development are outlined.

MATERIALS AND METHODS

Establishment and maintenance of cell lines

The ES cell line ES-D3 was derived from eight 129/Sv +/+ 4-day blastocysts, day of plug detection being set at 1 day of embryonic development. The ES-D3/4 and ES-D3/7, and ES-D3/10/5 cells are first- and second-order colony subclones, respectively, of the ES-D3 cells. ES-632 cells are a single-cell subclone similarly established from C57BL/6 blastocysts. After approximately 1–2 days of culture on a feeder layer of BALB/c 16- to 18-day embryonic fibroblasts (generously provided by Dr U. Koszinowski, Bundesforschungsanstalt für Viruskrankheiten der Tiere, Tübingen, W. Germany, see also Wobus, Holshausen, Jäkel & Schöneich, 1984) in Nunclon Delta SI tissue culture dishes, the inner cell mass cells were picked out, mechanically dissociated by gentle pipetting and transferred to a new feeder layer. Embryonal medium, consisting of 10 % foetal calf serum, 10 % newborn calf serum and 0·1 mM-β-mercaptoethanol in DMEM (Robertson *et al.* 1983), was changed every 2 days and the ES cells were transferred to new feeder layers about twice weekly. Embryonal medium was used during the establishment of all ES cell lines. After the lines were stable, they were maintained in the undifferentiated state on feeder layer cells. The maintenance medium was 15 % foetal calf serum in DMEM to which β-mercaptoethanol was added to 0·1 mM. The feeder layers were produced by treating the embryonic fibroblasts with 10 μg.ml^{-1} mitomycin-C for 3·5 h. The feeder layer cells were plated at approximately 5×10^6 cells per 90 mm tissue culture dish.

Cell culture under differentiation conditions

All ES and EC cells were cultured in the absence of embryonic fibroblasts in standard medium (15 % foetal calf serum in DMEM) either in tissue culture dishes, or in suspension in bacterial dishes or bottles placed on a rotary shaker. Cells were cultured in tissue culture dishes either under monolayer conditions using Falcon or hydrophilic petriperm (Heraeus) dishes at approximately 2×10^5 cells.ml^{-1} or under micromass culture conditions in which 10 μl droplets of 20 000 cells each were added to 24-well tissue-culture dishes (Costar) or to hydrophilic petriperm dishes. After allowing the cells to attach for 4 h the dishes were gently flooded with medium. Suspension culture in bacterial dishes (Greiner) also contained 2×10^5 cells.ml^{-1}. Suspension culture on a rotary shaker was performed at 70 r.p.m. with 20 mM-Hepes-buffered standard medium with approximately 10^5 cells.ml^{-1}. No significant differences could be found in the developmental potentiality of ES cells between the two types of suspension culture, or between monolayer and micromass cultures. 'Days of culture' will refer to the days of culture after switching the cells to differentiation conditions.

Karyotype

Chromosome spreads were performed as described (Triman, Davisson & Roderick, 1975) using the modifications kindly provided by S. Adolph (Klinische Genetik der Universität Ulm, W. Germany). Before spreading onto microscope slides pretreated with ethanol/ether (1:1), cells were treated 1–2 h with 10 μg.ml^{-1} colcemid, 10–15 min with 0·56 % KCl and 10, 20 and 39 min successively with methanol/acetic acid (3:1) at 4 °C. G-banding was done as described (Seabright, 1971).

Histology and immunofluorescence

Indirect immunofluorescence tests with the monoclonal antibody against trophectodermal cytokeratin-like filaments TROMA-1 (Brûlet, Babinet, Kemler & Jacob, 1980) and benzidine staining of erythrocytes in blood islands were performed on methanol-fixed (−20 °C, 10 min) cryostat sections of cystic embryoid bodies. The anti-mouse macrophage monoclonal antibody (MAS 034, Sera-Lab) was used in indirect immunofluorescence tests on the easily dissociable cells from mechanically disrupted cystic embryoid bodies. After pipetting the cystic embryoid bodies in and out of a Pasteur pipette several times, the single cells were centrifuged onto a gelatine-

coated microscope slide using a cytocentrifuge (Cytospin 2, Shandon) and fixed with 4% paraformaldehyde (4 °C, 10 min).

Immunoprecipitation and gel electrophoresis

Immunoprecipitation and electrophoresis of the fluid content of the *in vitro* cystic embryoid bodies was done by using the *Staphylococcus aureus* procedure of Kessler (1975) as applied by Vestweber & Kemler (1984). Cystic embryoid bodies were incubated in methionine-poor standard medium in the presence of [^{35}S]methionine (50 μCi.ml^{-1}) for 18 h after which the cavity fluid was collected either with a small Hamilton syringe or by gently breaking the cavities open and washing out the contents. Alphafoetoprotein (AFP) antiserum and affinity-purified anti-transferrin were kindly provided by E. Adamson (La Jolla Cancer Research Foundation, California) and were used at 20 μg.ml^{-1} (IgG fraction) and 3 μg.ml^{-1}, respectively, in the immunoprecipitations.

Blood cells for isoelectric focusing of haemoglobins were isolated and treated according to a modification of the method described by Cudennec, Delouvee & Thiery (1979). Briefly, adult 129/Sv blood cells were washed once in PBS, resuspended in 0·25 M-sucrose (containing 1% (v/w) trasylol (Sigma) and 0·1% KCN), centrifuged, and the cell pellet lyophilysed. Entire day-11 visceral yolk sacs (129/Sv) and blood-island-containing ES-D3 *in vitro* cystic embryoid bodies were ruptured and rinsed several times in PBS before resuspension in the above buffer. The 0·12 mm-thick isoelectric focusing gels were prepared and run on a flat-bed gel apparatus (LKB-Ultrophor) as described (LKB technical bulletin, modified by Dr Peter Symmons, Max-Planck-Institute for Developmental Biology, Tübingen, W. Germany) using pH 7–9 ampholines (Serva).

Electron microscopy

Samples were fixed for 2 h in 2·5% glutaraldehyde in PBS, postfixed 1 h each in 1% osmium tetroxide in PBS and 2% uranyl acetate in 70% alcohol, embedded in Epon, sectioned, contrasted 8 min at 25 °C in lead citrate in an LKB 2168 Ultrostainer and viewed with a Siemens Elmiskop 102 electron microscope at 80 kV.

RESULTS

Establishment and maintenance of the embryonic stem cells

After approximately 1–2 days of culturing 129/Sv+/+ blastocysts on a mitomycin-C-treated embryonic fibroblast feeder layer, clumps of inner cell mass cells from eight blastocysts (Fig. 1A) were picked out, mechanically dissociated by pipetting and transferred to a new feeder layer. In our hands 129/Sv ES cell lines could be established from approximately 5% of the attached blastocysts and C57BL/6 cell lines from about 10%. As long as the cells were maintained on a dense layer of feeder cells and replated every two days on a new feeder layer, differentiation was inhibited. This was determined morphologically (Fig. 1B) and by immunofluorescence with antibody markers specific for undifferentiated and differentiated embryonic cells (Kemler *et al.* 1981; Kemler, 1981; not shown). When carefully maintained in culture in the undifferentiated state as described above, ES cell lines can be kept in culture from 3 months to a year and can be freeze thawed several times without any apparent loss in developmental potential.

If the cells were grown on tissue culture plates without a feeder layer, differentiating, substrate-attached cells began growing out from the undifferentiated cell

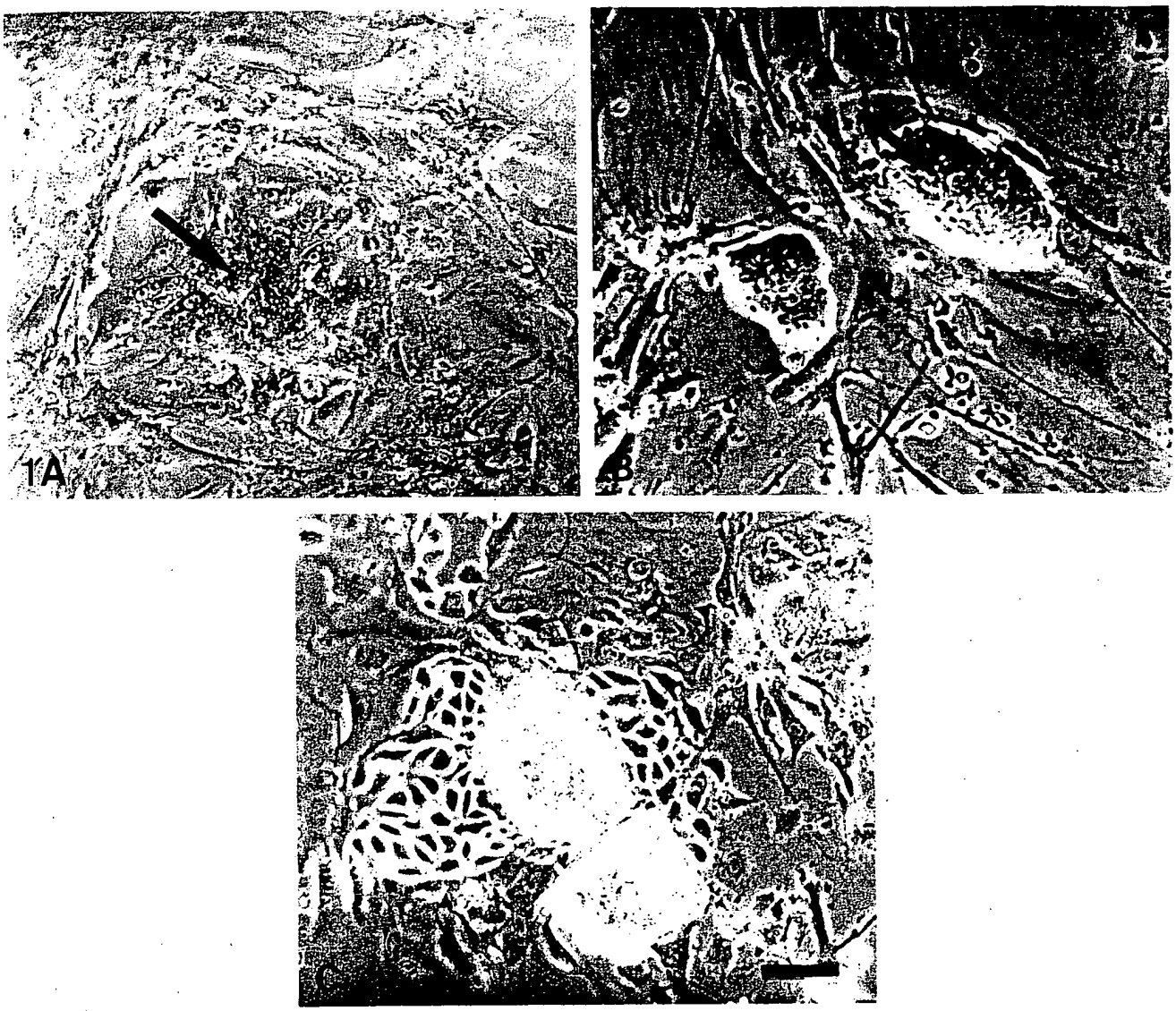


Fig. 1. ES cells during the establishment of cell lines, during maintenance in the undifferentiated state, and under differentiation conditions. 4-day 129/Sv blastocysts were allowed to attach to an embryonic fibroblast feeder layer. (A) Attached blastocysts when the inner cell mass cells (arrow) are removed and transferred to a new feeder layer after 2 days of culture. (B) Clumps of undifferentiated cells (arrow) being maintained on a feeder layer. (C) Differentiating ES-D3 cells after 2 days of culture on a gelatin-coated tissue culture dish in the absence of feeder layer. (Gelatine treatment is not necessary for ES cell differentiation.) Note the flat, differentiated cells growing out from the stem cell clumps. The few feeder layer cells remaining after transfer of ES cells to feeder-layer-free dishes are not sufficient to prevent differentiation. A–C: Bar = 100 μm.

clumps within 24 h (Fig. 1C). In all experiments the various ES cell lines seemed to behave identically. The blastocyst-derived cell lines have normal diploid karyotypes. Forty chromosomes were found in 62 % and 45 % of the cells, respectively, and both cell lines were XY. The G-banding pattern from one cell was analysed and revealed no translocations or metacentric chromosomes (Fig. 2).

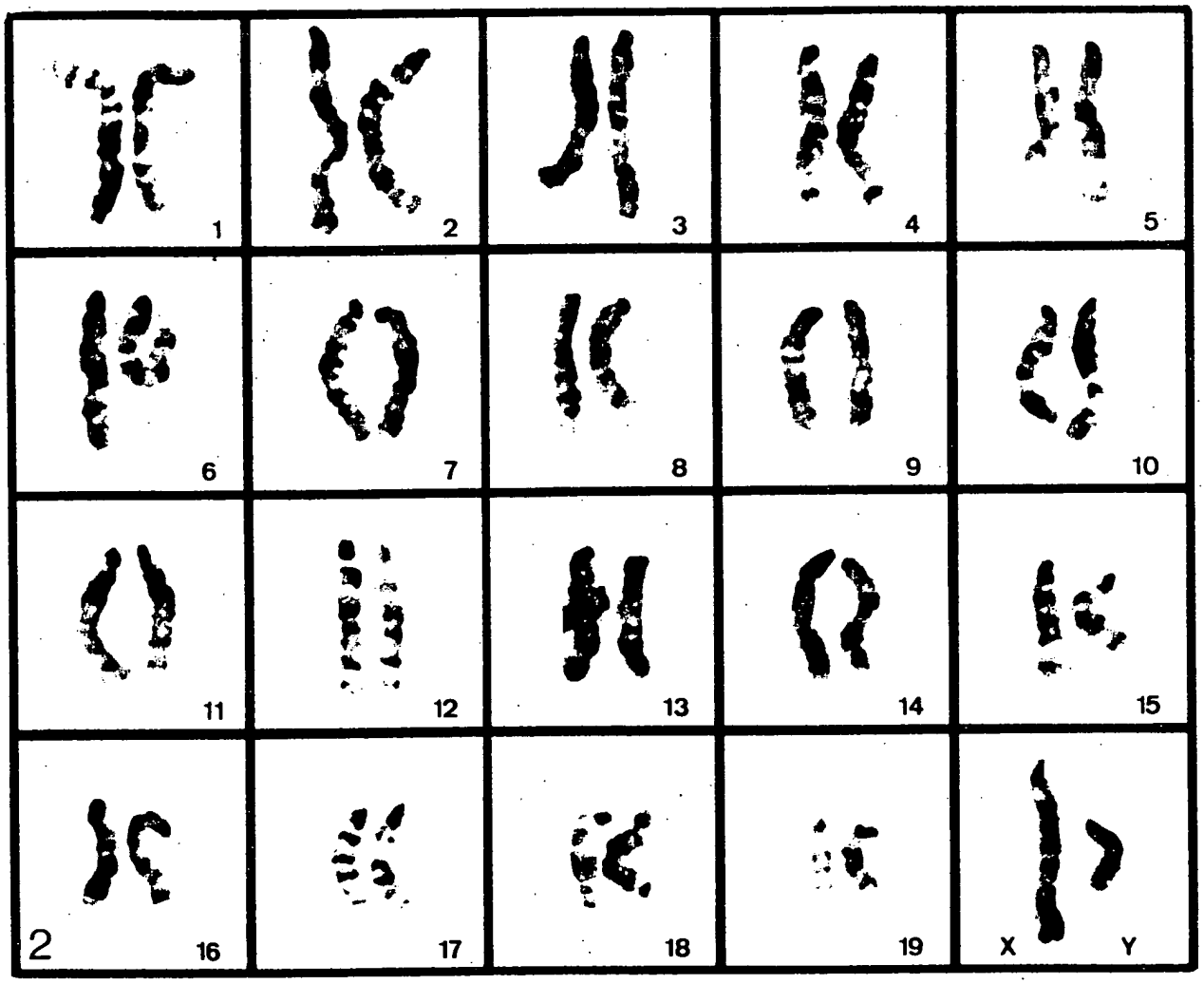


Fig. 2. Karyotype of a typical ES-D3/10/5 cell which has a normal diploid set of 40 chromosomes, has an XY constitution and contains no detectable abnormalities. ES-D3 cells: Out of 31 metaphase cells 62 % had 40, 25 % less than 40 and 13 % more than 40 chromosomes. ES-D3/10/5 cells: Out of 49 metaphase cells 45 % had 40, 32 % less than 40 and 22 % more than 40 chromosomes.

In vivo *formation of tumours*

To demonstrate that the ES cells could differentiate into products of all three germ layers, they were injected either subcutaneously or intraperitoneally into syngeneic mice, and the resulting tumours were analysed. When injected subcutaneously, solid teratocarcinomas were formed which contained large vacuoles enclosed by ciliated epithelial cells (preliminary evidence suggesting that these vacuoles have similarities to the brain ventricles), cross-striated muscle, cartilage, calcified cartilage, melanocytes and keratin sworls (not shown). When injected intraperitoneally, a mixture of mesenterically adherent solid tumours and unattached cystic embryoid bodies were formed. The latter contained outer and inner epithelial layers with areas of mesoderm in-between. The mesodermal areas

contained blood islands with embryonic haemoglobin-containing erythrocytes (see Fig. 8B for example) and contracting embryonic heart muscle cells (not shown). Immunofluorescence tests on cryostat sections of the cysts (not shown) showed that the cystic cavities contained large quantities of AFP.

In vitro *cultures*

In all of the experiments described below differentiating cells were cultured in standard medium without the addition of factors or inducers of any kind. Regardless of the type of culture (suspension (bacterial dishes or rotary shaker) or monolayer) most of the cells formed aggregates. If the aggregates were allowed to attach to the substrate (or remained attached), cells proliferated out from them along the substrate and differentiated into a wide variety of structures morphologically

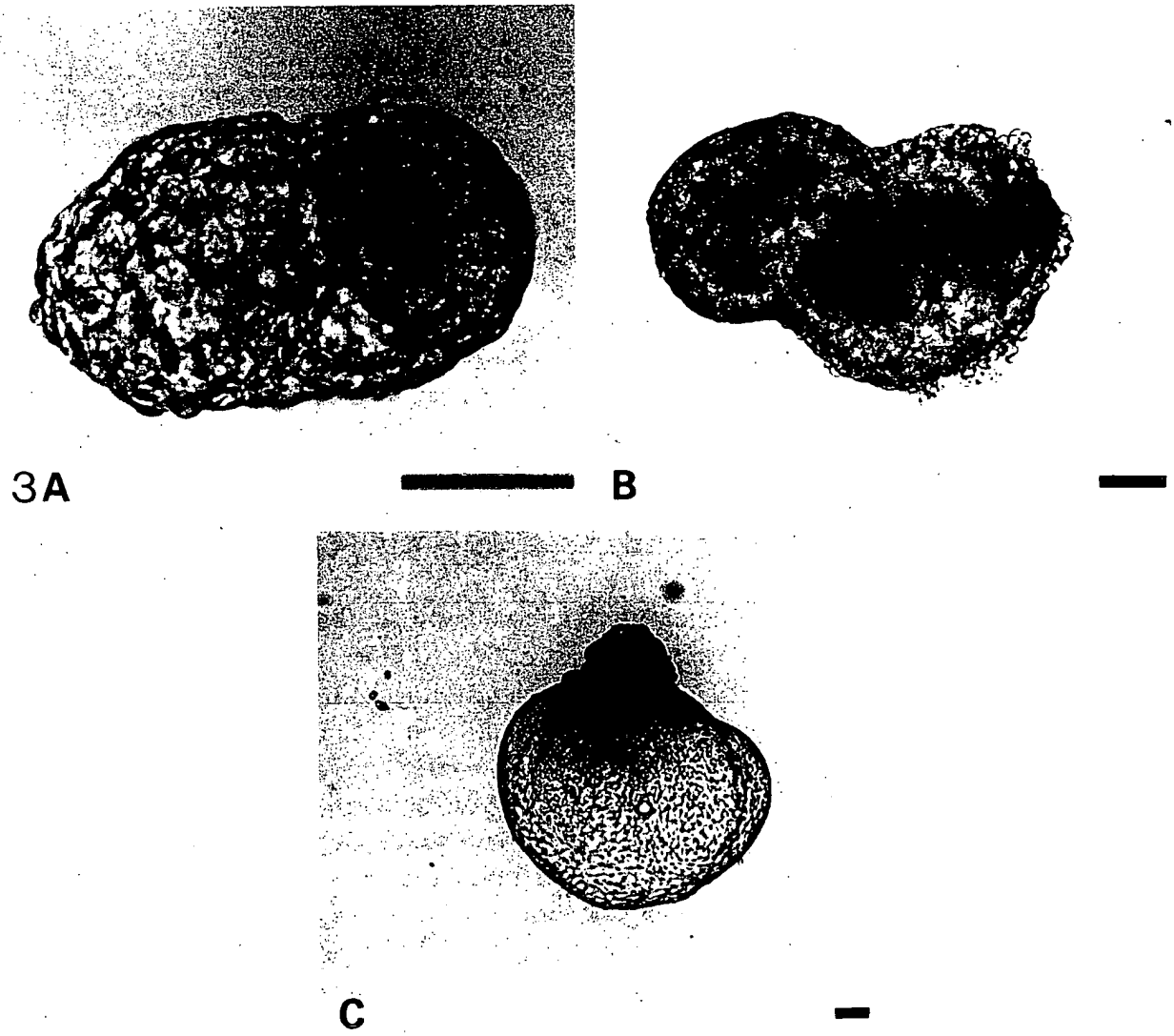


Fig. 3. Embryoid body development. ES-D3 cells were cultured under differentiation conditions in bacterial dishes for 4 days (A), 8 days (B) or 11 days (C). Bars = 100 μm.

identified at the light or electron microscopic levels as glandular, heart (see Fig. 5, for example), skeletal and smooth muscle, cartilage, nerve cells, keratin sworls, melanocytes and embryoid bodies (not shown). If, however, the aggregates were maintained in suspension, they developed only into embryoid bodies.

After 5 days of culture about 60 % of the embryoid bodies had developed into structures with an outer layer of endoderm bordered by a basal lamina within which the inner cells had condensed into a layer of columnar ectoderm-like cells. Many of these complex embryoid bodies were found to be polarized into two parts and appeared to be similar to the egg-cylinder stage of the 5-day embryo (Fig. 3A). Whether the two portions correspond to the extraembryonic and embryonic parts of the egg-cylinder-stage embryo is unclear. The markers we employed do not clearly distinguish between embryonic visceral on the one hand and extraembryonic visceral, primitive or parietal endoderm on the other. No trophoblast giant cells were ever seen during the entire culture period. During the next few days of culture there was a great deal of growth (compare Fig. 3A to 3B). By 8 days of culture an endodermal transition to the visceral type occurred along with the transition from complex to cystic embryoid bodies. After approximately 11 days of culture, many cystic structures were present (Fig. 3C) which looked similar to 8- to 10-day yolk sacs. After 3 weeks of culture, most developmental processes as well as growth had ceased, even though the cystic structures were viable for several more weeks.

Identification of cystic embryoid body as visceral yolk sac

The production of AFP and transferrin is characteristic of visceral yolk sac endoderm (Dziadek & Adamson, 1978; Adamson, 1982). Consequently, the fluid content of [^{35}S] methionine-labelled cystic embryoid bodies was electrophoretically analysed for total content (Fig. 4, lane 2) and by immunoprecipitations with anti-AFP and anti-transferrin (Fig. 4, lanes 3 and 4; the respective immunoprecipitations from 12-day embryonic visceral yolk sac: lanes 5 and 6). The total protein composition of the cavities consisted predominantly of AFP and transferrin with a few minor proteins of approximately 25 000, 45 000 and 300 000 relative molecular mass (M_r), presumably apolipoproteins A-I, A-IV and B, respectively (see Shi & Heath, 1984; Meehan *et al.* 1984) the 300 000 M_r protein which was precipitated by anti-transferrin was not precipitated from the labelling medium of embryonic visceral yolk sac cells (Fig. 4, lane 6). Electron micrographs of the outer endoderm cells of the cystic embryoid bodies (not shown) revealed many microvilli, electron-transparent cytoplasmic vesicles and junctional complexes as previously reported for EC cell cystic embryoid bodies (Martin *et al.* 1977), all of which are characteristic of visceral endoderm. Immunofluorescence tests on cryostat sections of cystic embryoid bodies using monoclonal antibodies TROMA 1 (positive for visceral and parietal endoderm, Kemler *et al.* 1981) and TROMA 3 (positive only for parietal endoderm, Boller & Kemler, 1983) (not shown) were consistent with the findings that the endodermal layer of the cystic bodies consisted predominantly of visceral yolk sac.

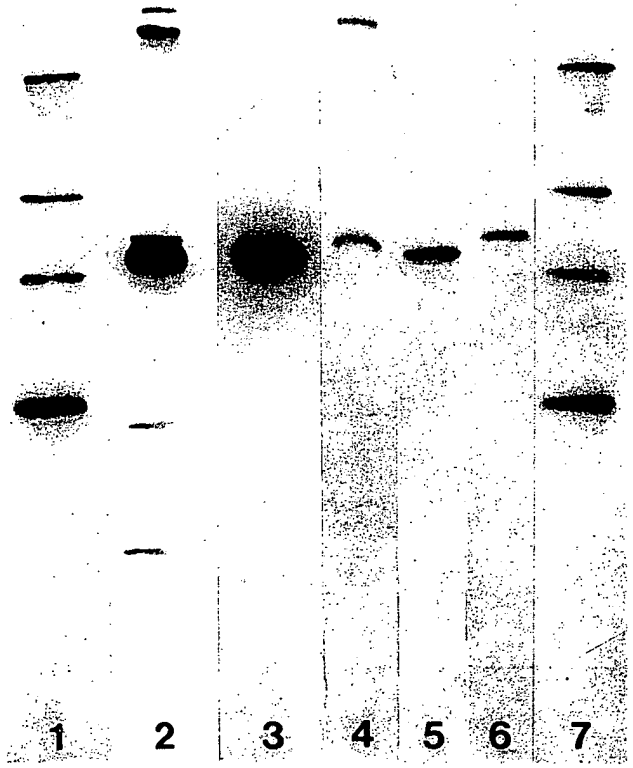


Fig. 4. SDS–PAGE and immunoprecipitation analysis of the protein content of ES cell cystic embryoid body cavities. Large, 2–4 mm in diameter ES-D3 or ES-D3/10/5 cystic embryoid bodies cultured for 2–4 weeks in bacterial or hydrophobic petriperm dishes (lanes 2–4), or freshly prepared 12-day visceral yolk sac (lanes 5, 6) were incubated with [^{35}S] methionine. Total labelled protein from cystic cavities (lane 2). Immunoprecipitation of cavity content (lanes 3,4) or labelled culture supernatant (lanes 5,6) with anti-AFP (lanes 3,5) or anti-transferrin (lanes 4,6). Note the high relative molecular mass protein coprecipitated by anti-transferrin from the cystic cavity contents but not from embryonic visceral yolk sac culture supernatant. Lanes 1, 7: protein relative molecular mass markers myosin heavy chain, 200 000; phosphorylase, 97 000; albumin, 68 000; and ovalbumin, 45 000.

Myocardium

After at least 8 days of suspension culture about one third of the ES cell cystic embryoid bodies began rhythmically contracting in areas where their surface was quite thick. Identically contracting structures could be found in micromass cultures and were analysed with respect to tissue organization and muscle type. Micrographs of video sequences of a highly organized beating structure (Fig. 5A, B) show the relaxed and contracted phase, respectively, of one contraction. The arch-shaped ridges (arrows) are phenotypically analogous to myocardium. An aggregate of cells was trapped in the endocardial-like cavity and moved back and forth during the contraction (arrowheads). Also associated with this structure were endothelial capillaries some of which also contained trapped cells which moved with the contractions (not shown). Electron micrographs showed that the muscle cells inside

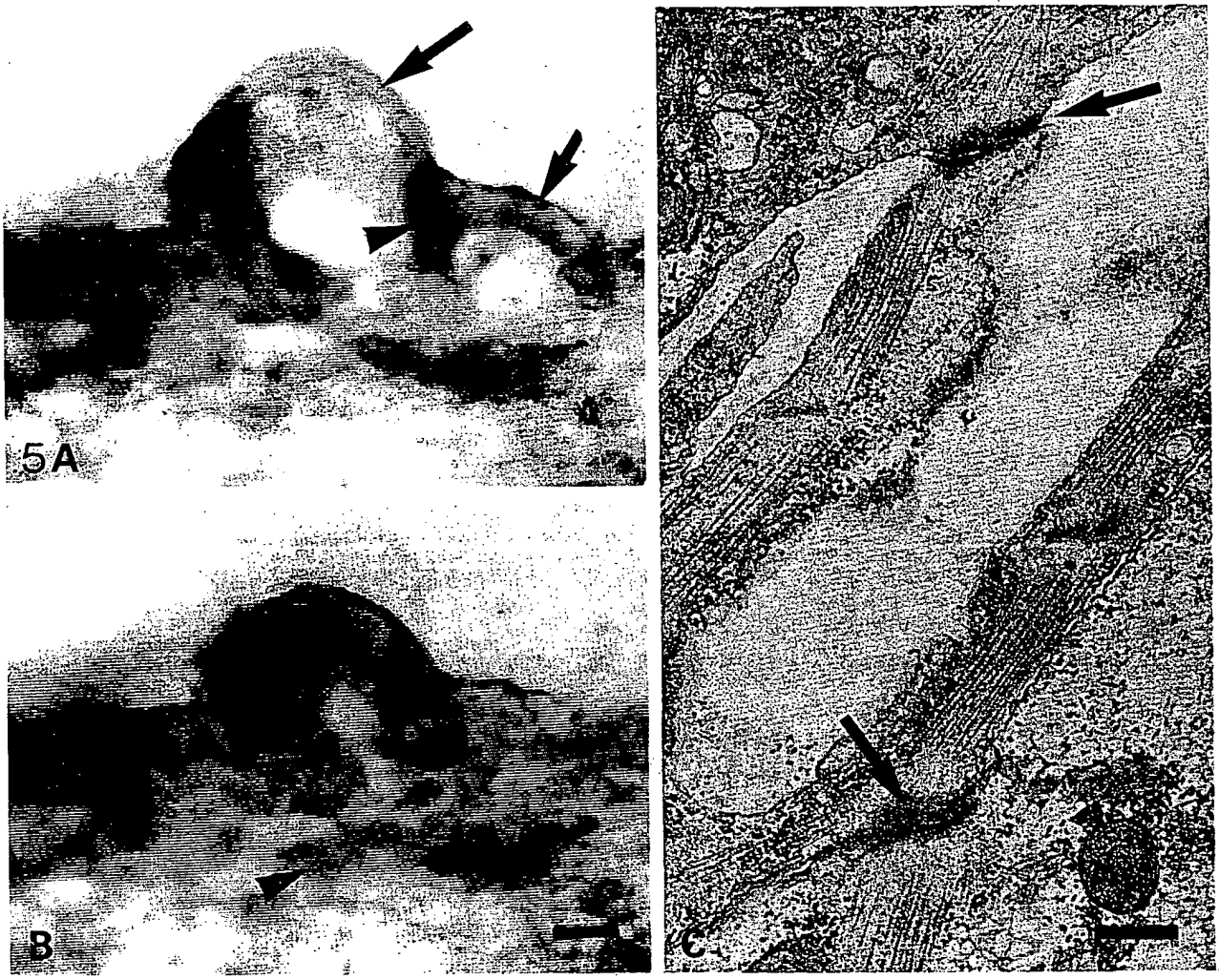
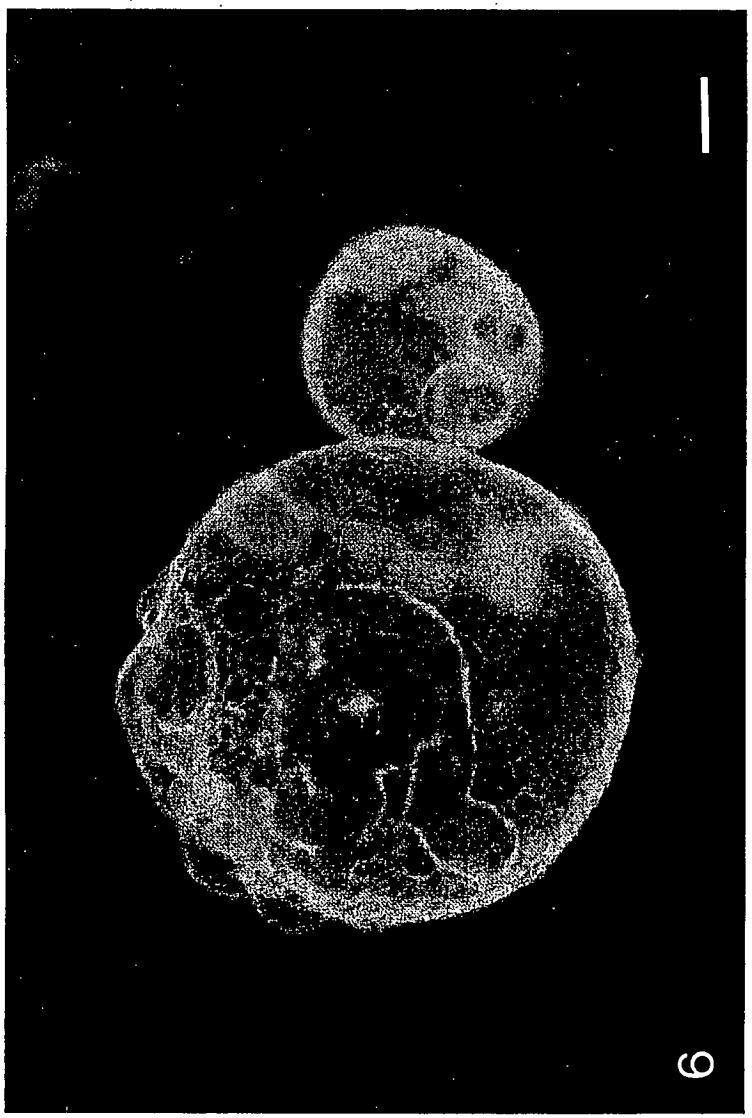
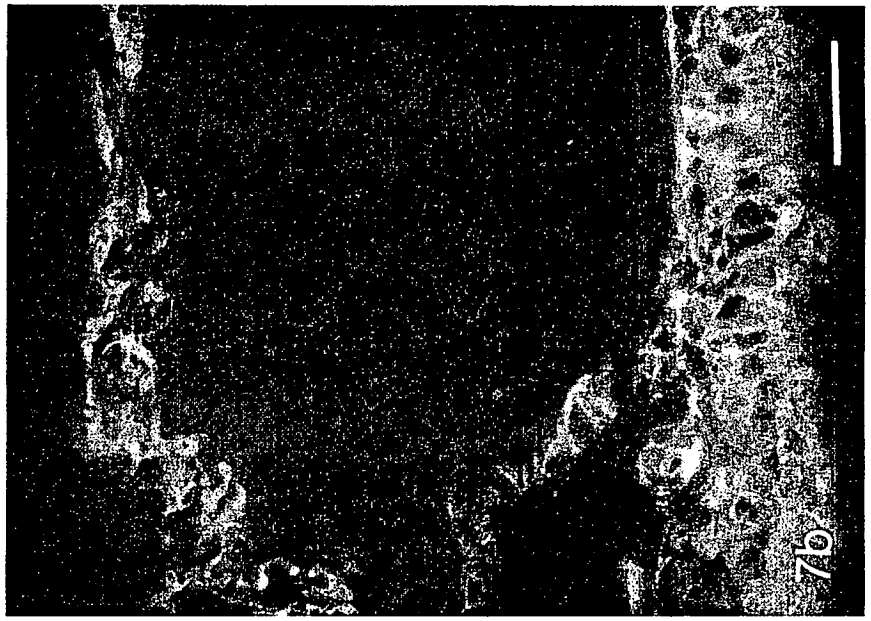


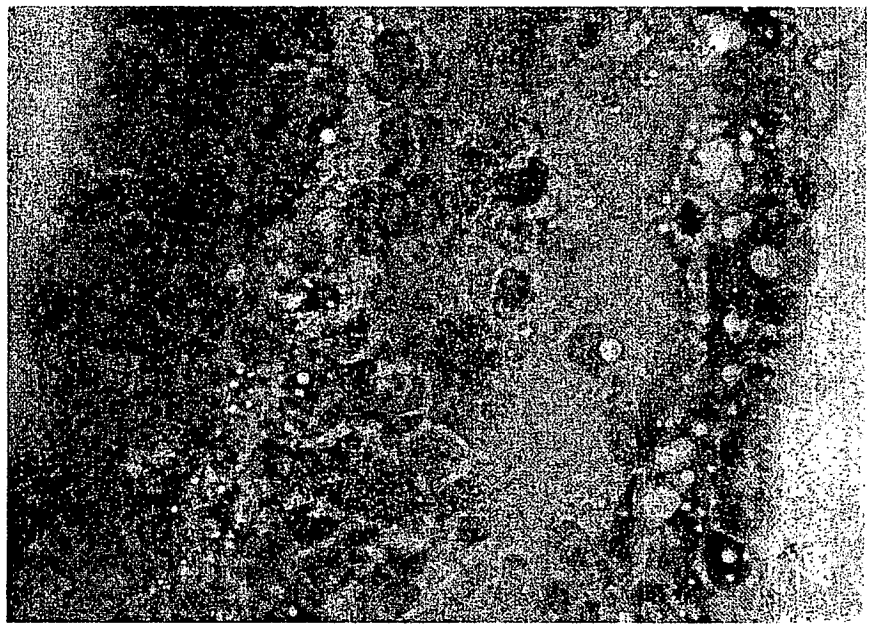
Fig. 5. Video micrographic and electron microscopic analysis of ES myocardial cells. (A,B). Polaroid photographs of video sequences of the relaxed and contracted phases, respectively, of one contraction. ES-D3 cells were grown in micromass culture. Myocard-like ridges (arrows) can be easily recognized in the relaxed phase of the contraction. Inside the endocard-like cavity a cell aggregate can be seen (arrowheads) which moves about 30 μm during the contraction. (C) Electron micrograph of ES-D3 cells which had been rhythmically contracting. The cells had been cultured for 4 days in suspension followed by 17 days on hydrophilic petriperm dishes. Note the intercalated disks which lie at the myofibrillar Z-bands of adjacent cells (arrows), structures characteristic of cardiac muscle cells. A,B: bar = 100 μm. C: bar = 0·5 μm.

Fig. 6. Dark-field photograph of ES cell cystic embryoid body. ES-D3/10/5 cells were cultured for 12 days on bacterial dishes. Besides the red blood islands, mesodermal thickenings (white areas) as well as endodermal subcompartmentalization can be seen. Bar = 200 μm.

Fig. 7. Histological and immunofluorescence analysis of ES cell cystic embryoid body blood islands. Cryostat sections of one blood-island-containing cystic embryoid ascites tumour removed 4 weeks after intraperitoneal injection of 5×10^6 ES-D3/10/5 cells. (A) Benzidine stain. In the inner (top) and outer (bottom) endothelial cell layers, only the nuclei are stained. Between these layers the cytoplasm of the erythrocytes (arrow) are stained as well. (B) Indirect immunofluorescence with TROMA 1. Endothelial cells are positively stained and mesodermal cells, including erythrocytes, are unstained. A,B: Bar = 50 μm.







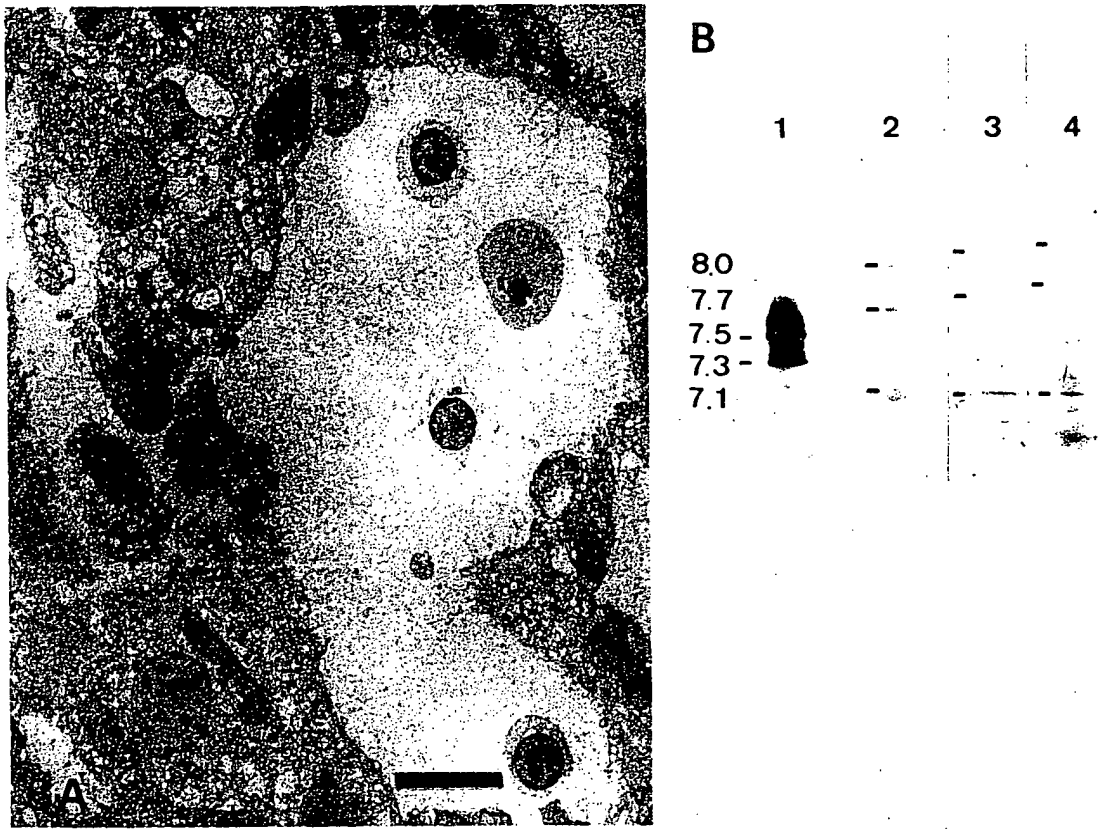


Fig. 8. Characterization of erythrocytes from ES cell cystic embryoid bodies and cystic tumours. (A) Electron micrograph of a blood island in an ES-D3/10/5 cystic embryoid body after 13 days of culture in a hydrophobic petriperm dish. The blood island consists of an endoderm-bordered cavity which contains unattached, nucleated erythrocytes typical of all yolk sac-derived red blood cells. (B) Isoelectric focusing of haemoglobin from three different gels (lanes 1 and 2, lane 3, and lane 4). The pH values indicated at the side are designated by a small line at the left side of each gel. Lane 1: adult globin chains from 129/Sv blood focus at pH 7·5 and 7·3. Lanes 2 and 3: embryonic globins from disrupted and washed visceral yolk sacs of 11-day 129/Sv embryos focus at pH 8·0, 7·7 and 7·1. Lane 4: embryonic globins found in disrupted and washed *in vitro* cystic embryoid bodies after 14 days in culture in bacterial dishes. The cells were cultured the first 10 days in standard medium and the last 4 days in 20 % foetal calf serum in Iscove's modified Dulbecco's medium. Benzidine reaction (lanes 1 and 2) and Coomassie blue staining (lanes 3 and 4). The cystic embryoid bodies were electrophoresed in their entirety in order to minimize the loss of erythrocytes. The visceral endodermal cells which greatly outnumber the erythrocytes in our samples are probably the source of the non-haemoglobin bands in lanes 2–4. Bar = 10 μm.

the myocard-like structures contained intercalated disks (Fig. 5C) which are heart and somitic myotome-specific intercellular junctions found where Z-bands of the apposing myofibrils of adjacent cells come together. The muscle cells could continue beating for more than a week. The morphological development of the *in vitro* beating structures was usually complete by the time the contractions were first observed.

Visceral yolk-sac-derived blood islands

At the light microscopic level red areas could be detected just under the surface of approximately 1 % of the cystic embryoid bodies after 12 days of suspension culture (Fig. 6). Closer examination of similar blood islands found in cystic tumours using benzidine (Fig. 7A; stained erythrocyte cytoplasm being indicated by arrow) and fluorescence staining with TROMA 1 (Fig. 7B) revealed a pocket of blood cells surrounded by two endothelial layers. An electron micrograph (Fig. 8A) shows that the blood island erythrocytes of an *in vitro*-formed cystic embryoid body were nucleated — characteristic of the blood cells of embryonic visceral yolk sac. The haemoglobins of blood island cells in *in vitro* cystic embryoid bodies were determined by isoelectric focusing to be embryonic (Fig. 8B, lane 4; control adult, lane 1; and control embryonic, lanes 2 (benzidine) and 3 (Coomassie blue)). The double control shows that Coomassie-blue staining can also be used to detect haemoglobins in these structures. In cystic tumours the blood islands also contained exclusively embryonic haemoglobin (not shown), thus demonstrating that host erythrocytes had been excluded from the cystic tumours. As may be the case in the mouse embryo, the blood islands in the *in vitro* cystic embryoid bodies usually disappeared after 2–6 days.

In order to increase the frequency of appearance of blood islands, culture conditions used for blood stem cell cultures (Iscove's modified Eagle's medium) and for mouse embryo *in vitro* cultures (20 % human cord serum instead of foetal calf serum; Hsu, 1979) were combined. These culture conditions increased the percentage of cystic embryoid bodies which contained blood islands from 1 % to 30 % (Table 1). Four different cell lines from two different mouse strains gave similar results. These data suggest that except for possible small quantitative

Table 1. *Blood island production in various differentiated ES cell lines.*

Mouse strain	Cell line	No. of exps.	Blood-island-containing cystic structures	
			FCS	HCS
129	ES-D3/7	5	3/165 (2%)*	78/249 (30%)
129	ES-D3/10/5	1	1/281 (1%)	22/218 (19%)
129	ES-D3/4	1	0/102 (0%)	19/76 (25%)
C57	ES-632	2	3/34 (10%)	16/48 (33%)

ES cells were cultured in bacterial dishes in the absence of embryonic fibroblasts and in standard medium for 10 days. On day 10 the medium was changed to Iscove's modified Eagle's medium containing either 20% foetal calf serum (FCS) or human cord serum (HCS). Every 2 days thereafter each cystic, visceral yolk sac structure was examined under dark-field stereo optics. Each cystic structure containing one or more blood islands was scored as one. The scores from days 14–18 of culture were combined. Data were taken only from experiments in which both culture conditions were used.

* Number of blood-island-containing cystic embryoid bodies/total number examined.

differences between ES cell lines, they all display qualitatively similar developmental potentialities.

Immunofluorescence tests with a monoclonal antibody specific for macrophage cells (Fig. 9A and B) suggest that the cystic embryoid bodies may also contain

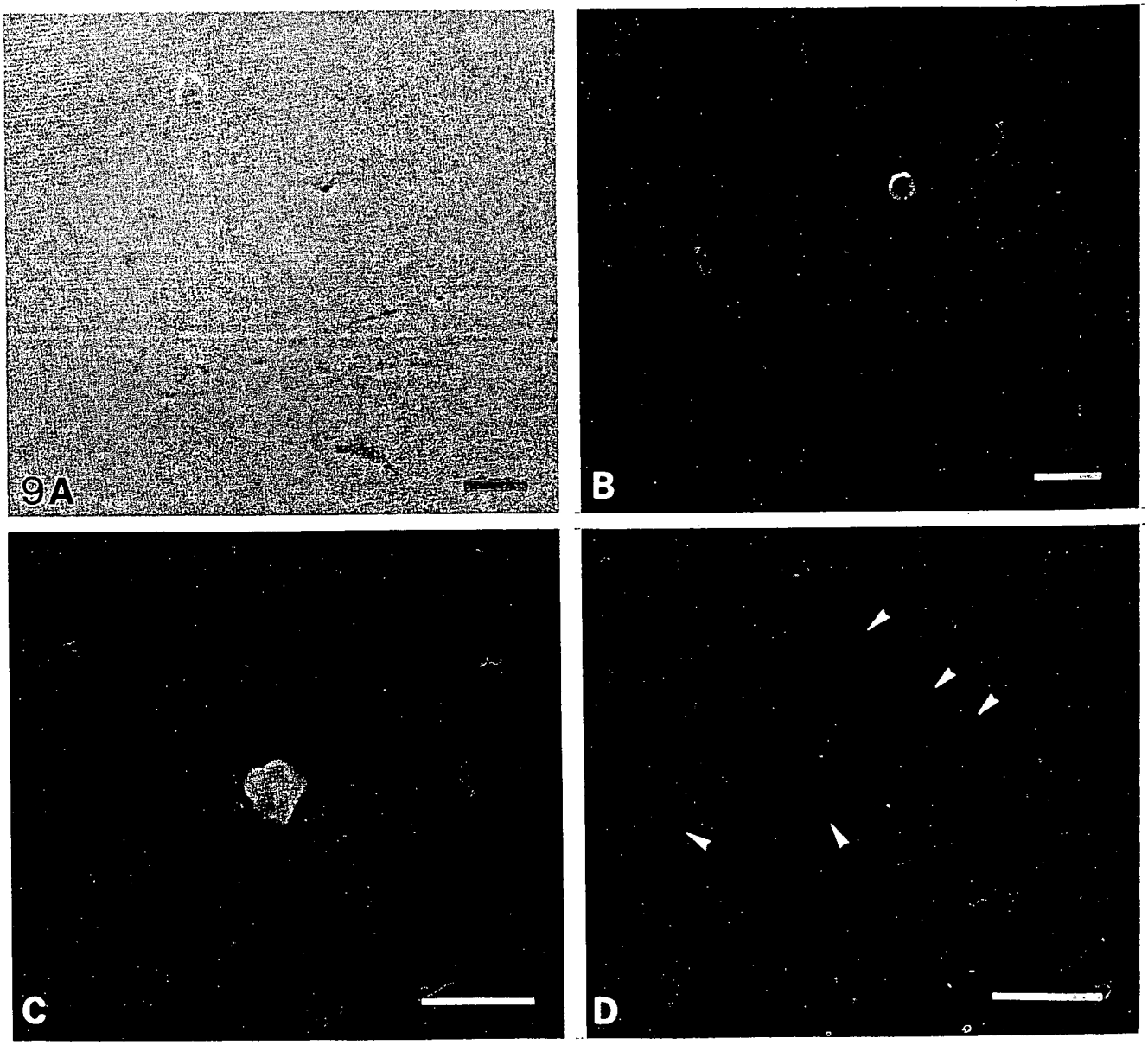


Fig. 9. Indirect immunofluorescence test with anti-macrophage monoclonal antibodies. (A,B) Positive control: Mouse peritoneal fluid was placed in tissue culture. After 2 days of culture, the attached cells were fixed and indirectly stained with antibody. Note the fibroblastic and non-fibroblastic cells which do not stain. (C,D) Cells were mechanically dissociated (see Materials and Methods section) from 18 day *in vitro* ES-D3 cystic embryoid bodies and cytocentrifuged onto microscope slides. (C) Indirect immunofluorescence test with antibody. (D) Control: Fluorescent second antibody alone. Arrowheads indicate location of cells. (A) Phase contrast. B–D, Fluorescence. Bars = 50 μm.

macrophages (Fig. 9C). Preliminary results using methyl cellulose culture conditions also indicate the presence of macrophage colony-forming cells in the cystic embryoid bodies (Gordon Keller, Basel Institute of Immunology, Switzerland).

Comparison to EC cells

Two teratocarcinoma-derived cell lines have been reported to produce cystic structures which occasionally contained erythrocytes *in vitro* (Martin *et al.* 1977; Cudennec & Nicolas, 1977). We have compared a subclone (EC-PSA1/NG2) of one of them to our blastocyst-derived cell lines. The greatest difference between the ES and EC lines was of a quantitative nature (not shown). There appeared to be more mesoderm-derived structures in the ES cells, and although the teratocarcinoma-derived cells could form similar highly organized structures, the frequency with which they formed them was strikingly less.

DISCUSSION

There are four aspects of blastocyst-derived embryonic stem cell lines which together make them potentially useful as a model system for embryonic development. The first is that they can be established from individual blastocysts of nearly any genotype. This makes it possible to investigate interstrain differences which may become apparent during embryogenesis. ES cell lines have been used for the investigation of cells homozygous for a lethal recessive mutation carried by inbred mouse strains (Magnuson *et al.* 1982), of gross chromosome abnormalities such as metacentricity (Kaufman, Robertson, Handyside & Evans, 1983), and of parthenogenesis (Robertson *et al.* 1983). Other uses could be to investigate the effects of mono- and trisomy (Gropp, 1982) and to investigate whether the differences between mouse strains which do and do not form spontaneous teratomas at high frequencies are caused by differences in the embryonic cells or in their host environment. Investigations of this nature could be of potential importance to the problem of transformation.

The second aspect, the consistency from one cell line to the next with respect to the *in vitro* developmental process, is necessary if the homozygous mutant lines referred to above are to be fruitfully compared to their respective background lines. We have now closely observed six ES cell lines from three different genetic backgrounds as well as many of their subclones (single cell as well as colony subclones) and have found them all to be remarkably similar with respect to their developmental characteristics.

In light of their developmental consistency, the third aspect of ES cell lines, namely their ease of establishment, is of special significance. One now has the advantage of always being able to work with cells which are temporally close to the embryo. It is generally accepted that the more time an embryonic cell spends outside of the embryonic environment, the more likely it will be selected for growth in the new environment. Consequently, both EC and ES cells can be expected to

lose their pluripotent characteristics over time. The special advantage of the blastocyst-derived ES cells is that under such circumstances a new line can easily be made with the same original potentialities of the old.

The fourth aspect of ES cells which make them suitable as a model system for embryonic development is their *in vitro* expression of a large degree of developmental potential. Since ES cells are capable of forming germ-line chimaeras at a frequency much higher than teratocarcinoma cells (Bradley *et al.* 1984), one would also expect higher *in vitro* frequencies of well-developed embryonic structures. This can be realized through two types of culture conditions: under two-dimensional (substrate-attached) culture conditions ES cells can differentiate into a large variety of cell types (Martin, 1981; this report). Such conditions may be well suited for investigations into some of the determining events which lead to terminal differentiation, especially if humoral factors are involved. In three-dimensional suspension culture ES cells form highly organized cystic embryoid body structures which are in many respects analogous to postimplantation embryos. With these structures one should be able to answer more easily questions concerning 'development' of the embryo rather than simply 'differentiation' of cell types. They may also be suitable for studying the developmental regulation of the expression of genes, normal or altered, inserted into ES cells, thereby offering all of the analytical advantages of *in vitro* systems. It is on these highly organized cystic embryoid bodies that we have focused our attention in this report.

Comparison of ES cell development to that of the embryo and EC cells

The major proteins synthesized by the ES cell cystic embryoid bodies and secreted into their cavities are AFP and transferrin — two of the major products of the visceral yolk sac. It is noteworthy that other than these two proteins very few others are detectable. The minor proteins of approximately 25 000 and 45 000 M_r, but not the one of 300 000 M_r, have been observed previously in visceral yolk sac fluid (Adamson, 1982; Janzen, Andrews & Tamaoki, 1982). Recently, apolipoproteins of all three sizes have been found to be produced by visceral yolk sac endoderm (Shi & Heath, 1984; Meehan *et al.* 1984). We do not know why an approximately 300 000 M_r protein is coprecipitated by anti-transferrin serum though not recognized by this same serum in immunoblots. This protein could not be coprecipitated from embryonic visceral yolk sacs metabolically labelled *in vitro* and is therefore unlikely to be apolipoprotein B.

In the embryo blood islands appear within the mesodermal layer of the visceral yolk sac on day 8. These primitive erythrocytes are large, contain nuclei and synthesize primitive haemoglobins (Craig & Russell, 1964). *In vitro* erythropoiesis has been shown to occur in two teratocarcinoma-derived EC cell lines. The large, nucleated red blood cells produced by the cystic structures of EC-PSA1 (Martin *et al.* 1977) and EC-PCC3/A/1 (organ culture conditions; Cudennec and Nicolas, 1977) develop blood islands from mesodermal thickenings on the inner side of

endodermal vesicles. The EC-PCC3/A/1 blood cells contain embryonic haemoglobin (Cudennec, Thiery & Le Douarin, 1979).

Under standard culture conditions approximately 1 % of the blastocyst-derived ES cell cystic bodies contain islands of large, nucleated, embryonic haemoglobin-containing erythrocytes after two weeks in culture. A 30-fold increase in the percentage of blood island-containing cystic bodies can be induced by human cord serum. That these cells could make adult haemoglobin under appropriate conditions, however, cannot be ruled out. This is important in light of evidence that some sera used in culture (Stamatoyannopoulos, Nakamoto, Kurachi & Papayannopoulou, 1983) as well as other embryonic tissue (Cudennec *et al.* 1981; Ripoche & Cudennec, 1983; Labastie, Thiery & Le Douarin, 1984) can induce yolk sac erythrocytes to produce adult haemoglobin. The cystic embryoid bodies may also contain stem cells for macrophages. The existence of other stem cells of the haemopoietic lineage is presently under investigation. The presence of haemopoietic stem cells in the cystic structures may provide investigators with a purely *in vitro* model system for unravelling some of the complexities of the haemopoietic cell lineages.

In the embryo the first muscle cells to appear are in the myocardium and the somitic myotome. Whereas the myotome-derived muscle anlagen produce multinucleated cells, the myocardial cells remain by and large mononucleated and develop intercalated disks which serve to join the myofibrillar apparatus of adjoining cells (rev. by Manasek, 1973). Contractile protein isoforms also seem to follow this pattern in that cardiac isoforms are found both in myotome and myocard, but not in skeletal muscle (Toyota & Shimada, 1981; Sweeney *et al.* 1984). The only distinguishing characteristic then between the earliest myocardial and myotomal muscle is the rhythmic contraction of the primitive heart cells.

The production of beating muscle cells by two teratocarcinoma-derived cell lines has been reported. EC-PSA1 cells produce such cells both in monolayer culture (Martin *et al.* 1977) and in cystic embryoid bodies (our observations). EC-P19 cells, which require chemical inducers to differentiate, form beating structures in monolayer culture (McBurney *et al.* 1982). The development of associated endocardial tissue by these cell lines has not been described previously.

About one third of the cystic embryoid bodies produced by the blastocyst-derived cells develop rhythmically contracting, intercalated disk-containing myocardial cells. The associated endocardial tissue found in cultures of substrate-attached cells can also form in the cystic structures (not shown). These data show that ES cells have the potential to develop into several cardiac cell types in a well-organized manner, suggesting that they may be suitable for investigations of heart organogenesis.

It is pertinent to this discussion that only the embryonic and not the extra-embryonic portion of egg-cylinder-stage embryos can 1) form teratocarcinomas (Diwan & Stevens, 1976) or 2) develop *in vitro* into structures similar to those described here (Hogan & Tilly, 1981). Likewise, it is noteworthy that the structures

described in this report are similar to those produced by some of the earlier attempts at embryo culture (Hsu, 1972). These data are consistent with the chimaera experiments mentioned above (Bradley *et al.*, 1984) and lead to the conclusion that ES cells are in fact quite similar to the pluripotent cells of the blastocyst. We are confident that the developmental similarity of most of the ES cell lines produced, their ease of establishment, their ability to form highly organized structures analogous to those of the embryo, and their amenability to the production of interstrain variants, should provide investigators with new approaches to the study of embryonic development.

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New prospects for human stem-cell therapy in the nervous system

Clive N. Svendsen and Austin G. Smith

It would be of enormous benefit if human neural tissue could be generated *in vitro* as this would allow screening for neuroactive compounds, and provide a source of tissue for testing cellular and gene therapies for CNS disorders. It is now well established that pluripotent embryonic stem cells (ES cells) from the mouse can be propagated in culture and differentiated into a range of tissues, including neuronal and glial cells. In other studies, more-restricted neural stem cells have been isolated from both the developing and adult rodent brain. Current reports now describe similar pluripotent and neural stem cells cultured from human embryos. While the exact nature of these cells continues to be explored, they can be grown for extended periods of time while retaining the capacity for neuronal and glial differentiation. In some cases, they have been shown to integrate into the developing or damaged adult brain. This article reviews their biology, with a focus on the possible links between ES-cell and neural stem-cell technologies, and the strategies used to isolate and expand defined cell populations.

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PHARMACEUTICAL or surgical interventions into neurodegenerative diseases have, in general, met with limited long-term success and are often compounded by serious side-effects. Whilst such avenues should continue to be explored and refined, a third approach, cell therapy, now warrants serious consideration. Current clinical trials suggest that the transplantation of dopaminergic neurones derived from primary human foetal tissues can have beneficial effects in patients with Parkinson's disease[1]. In one case, post-mortem data have shown good survival of dopaminergic neurones within a transplant, proving that these cells can survive and mature within the adult human brain[2]. Similar trials that involve cell therapy are being undertaken for Huntington's disease[3,4] and are being considered for myelin-related disorders[5]. However, human foetal tissues are extremely difficult to obtain in sufficient quantities for transplants. Generation of neural tissue *in vitro* would, therefore, be a key milestone towards developing cellular and gene therapies for disorders of the CNS.

The ability to propagate pluripotent mouse embryonic stem (ES) cells and then induce differentiation has enabled the production *in vitro* of tissues for functional studies, drug screens and experimental transplantation[6–8]. A previous article[9] has discussed the first report of neurones generated from mouse ES cells[10] and the possibility of using such tissues for transplantation. In the interim, a series of research papers and a number of stimulating reviews[11–17] have described some of the molecular mechanisms that underlie the proliferation and differentiation of rodent neural stem cells derived from the developing or adult CNS, which has provided the experimental groundwork for this new field. It would now appear that the areas of embryonic and neural stem-cell biology will become increasingly more interwoven over time.

Until recently, ES cells could only be generated from mice. However, two reports have now appeared that provide the first evidence to show the derivation of pluripotent stem cells from human embryos[18,19]. These

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Box 1. Applications of *in vitro* generation of human neural tissue

- Identification of inductive factors and mediators in human neurogenesis
- Determination of gene function in neuronal and glial differentiation
- Modelling neurodegeneration
- Pharmaceutical discovery
- Cell therapy
- *Ex vivo* gene therapy

cells retain the potential to differentiate into various tissue lineages after growth in culture, which offers the prospect of developing novel cell-transplantation therapies[20]. Meanwhile, in the neuroscience arena, human neural precursor cells were recently cloned[21], expanded for extended periods of time *in vitro*[22,23] and transplanted successfully into foetal rodent brain[24]. These findings on human tissues extend stem-cell research from being principally academic to being potentially medical. In addition, the applications for generating human neurones *in vitro* should not be overlooked (Box 1). In this context, *ex vivo* maturation of stem cells into terminally differentiated phenotypes with high fidelity to adult cells *in vivo* will be crucial for many purposes. These include their use as research tools for the genetic dissection of neuronal developmental and cell biology, and in the creation of new assays for pharmaceutical discovery, such as high-throughput screens for neuroprotective compounds.

Below, the emerging data on human stem cells is related to studies in rodents and to the ultimate goal of establishing cell and gene-delivery systems for CNS therapy. The ethical and regulatory issues associated with using such human tissues are summarized in Box 2.

Pluripotent embryo-derived stem cells in mice

The experimental investigation of mammalian embryonic development is complicated by the inaccessibility of the embryo *in utero*. This has resulted in efforts to isolate and propagate in culture the founder stem cells of the foetus. In mice, pluripotent stem-cell lines can now be established that produce derivatives of all

Box 2. Ethical and regulatory issues

The regulations governing research with human embryonic and foetal material differ between countries. In the UK, research on human embryos is regulated by the Human Fertilisation and Embryology Act (1990), which prohibits the propagation of an intact embryo beyond 14 days. Pluripotent stem-cell cultures are established by disaggregating embryos between 6 and 8 days. Research projects on these cells can only be conducted under a research licence issued by the Human Fertilisation and Embryology Authority. Neural stem-cell cultures are established from post-mortem foetal material. There are no statutory restrictions on research with post-mortem foetal material, provided ethical approval is obtained from the relevant authority and tissues are collected under guidelines set out in the Polkinghorne report and Department of Health guidelines.

Box 3. Characteristics of embryonic stem cells

- Non-transformed
- Indefinite proliferative potential
- Stable diploid karyotype
- Clonality
- Formation of multi-differentiated tumours (teratomas) on ectopic transplantation
- Multi-lineage differentiation *in vitro*
- Permissive for genetic manipulation
- Incorporation in chimaeras (not practical for human tissues)
- Germline transmission in chimaeras (not practical for human tissues)

three primary germ layers: endoderm, mesoderm and (neuro)ectoderm[6]. These ES cells are derived without the use of any immortalizing or transforming agents and are distinguished by a number of specific features (Box 3). The most-remarkable property of ES cells is their ability to reintegrate into the developing embryo. They can colonize all lineages to produce chimaeric animals that contain a mixture of ES-cell-derived and host-embryo-derived progeny in all tissues, including the germline[25]. The viability and fertility of ES-cell chimaeras demonstrates the intrinsically normal character of ES cells. They also establish the capacity of ES cells to give rise to functionally mature adult cell types.

Mouse ES cells can be derived from two sources. Conventionally, they are isolated from the undifferentiated epiblast cells present in the blastocyst prior to implantation[6]. Either whole blastocysts or isolated epiblasts are plated onto feeder layers of mitotically inactivated mouse-embryo fibroblasts alone or in the presence of the cytokine leukaemia inhibitory factor (LIF)[26]. After several days the expanded epiblast is gently dislodged, dissociated and re-plated. Colonies of stem cells can then emerge, although the incidence with which this occurs is highly dependent on the mouse strain. The second method for obtaining ES cells uses the primordial germ cells (PGCs) of the post-implantation embryo[27]. In this case, fibroblast growth factor 2 (FGF2) and stem-cell factor must be provided during the initial culture period. These factors, which act in concert with LIF, induce proliferation followed by de-differentiation or reprogramming of the PGCs. This results in their conversion to so-called embryonic germ (EG) cells that are phenotypically indistinguishable from blastocyst-derived ES cells.

Self-renewal of mouse ES cells is dependent on LIF or related cytokines that act to suppress differentiation[28]. Multilineage differentiation is induced by aggregation of the ES cells in the absence of LIF. The ES-cell aggregates form structures called embryoid bodies[29]. These embryoid bodies do not exhibit the morphogenetic organization of the embryo proper, but they do undergo similar cellular differentiation to generate an extra-embryonic yolk sac and foetal germ layers[30]. On re-attachment to a substratum, further differentiation proceeds in outgrowths from the embryoid body. The spectrum of cell types generated can be influenced by various parameters, such as cell numbers in the aggregates, the presence of growth factors and the addition of inducing agents, such as retinoic acid. Defined products include

cardiomyocytes, skeletal myoblasts, multiple haematopoietic cell types, endothelial cells, adipocytes and epidermal cells[8]. Differentiation of neuronal and glial cells in embryoid body outgrowths has been documented by several groups[10,31,32]. More recently, neural precursors have been isolated from embryoid bodies that can be expanded in culture using mitogens, and might resemble precursor cells isolated from the embryonic CNS, which are described in a later section[33,34].

Transplantation of ES cells

The differentiation of ES cells *in vitro* yields primarily embryonic or foetal cell types. In the context of cellular transplantation, the developmental plasticity and proliferative properties of lineage-restricted but still immature cells could be an advantage. It can be argued that naïve precursor cells might respond to inductive, trophic and migratory cues from the host micro-environment more appropriately than fully differentiated cells do. They could also show greater flexibility for integration into existing cytoarchitecture. In practice, however, adult tissue is probably unlikely to retain all the relevant instructive signals, and some degree of phenotypic commitment might be required prior to grafting. Examination of the transplantation potential of ES-cell progeny is only in its infancy at present. Cardiac and haematopoietic derivatives have been transferred into adult mouse heart and circulation, respectively. In both cases, long-lived grafts can be obtained with evidence of functional maturation of the donor cells[35,36]. Attempts to reconstitute the mouse haematopoietic system entirely from *in vitro* differentiated ES cells have not been successful to date[8,37].

The ability of ES-cell-derived neural cells to integrate into the developing brain has been investigated by *in utero* injection into the telencephalic vesicle of late gestation rat foetuses[38]. Animals were examined several days after birth and donor cells discriminated by staining with species-specific monoclonal antibodies and by *in situ* hybridization detection of a mouse major satellite sequence. Both neuronal and glial derivatives of injected cells were found to be widely distributed in various brain regions. Cell morphology differed according to the location, which suggests the appropriate specification of neuronal subtypes, though no molecular markers were investigated. In a second study, Isacson and colleagues[39] injected differentiated ES cells intracerebrally into adult mice and immunosuppressed rats. They found extensive projections from the graft sites into white and grey matter, with evidence of both serotonergic and dopaminergic neurones plus glia. Thus, it appears that ES-cell-derived neural cells will be able to survive, migrate and differentiate in the intact brain.

However, a significant problem in using ES-cell progeny for transplantation has also been highlighted by these studies. The difficulty stems from our current inability to direct ES-cell differentiation efficiently. Existing differentiation protocols produce an assortment of cells from different lineages and of differing maturity. Consequently, the transplanted cell populations tend to be highly heterogeneous and the resulting grafts tend to be populated by multiple cell types. This can impede access of the donor neural cells to host tissue and might antagonize or suppress host-inductive signals. Yet more serious is the potential for any undifferentiated ES cells present to continue proliferating and produce teratomas or even malignant teratocarcinomas[38,39].

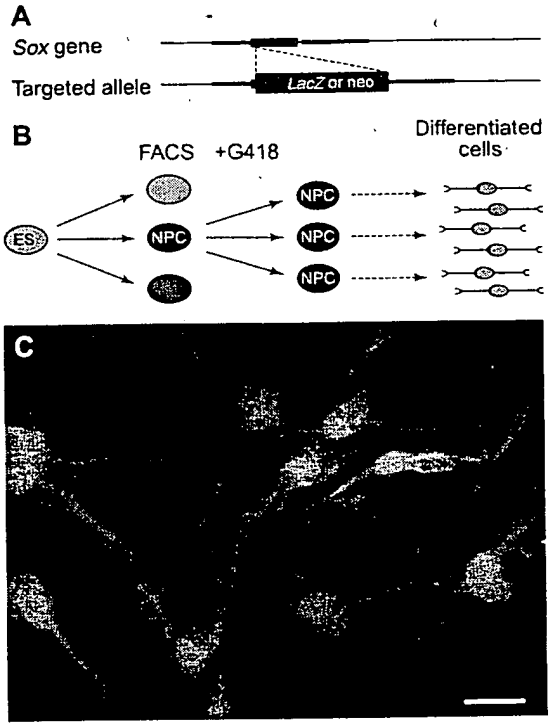

Fig. 1. *Schematic representation of the lineage selection strategy for isolating pure populations of neural precursor cells (NPC) from embryonic stem (ES) cells.* **(A)** *shows stage I. A selection marker [such as LacZ, which encodes a reporter enzyme, β-galactosidase or neomycin (neo), that confers G418 resistance] is integrated by homologous recombination into a gene, such as certain members of the Sox gene family, that is expressed exclusively in neural precursors. The marker can be designed to enable fluorescence-activated cell sorting (FACS) of expressing cells, for example β-galactosidase or green fluorescent protein (GFP), in addition to drug selection (+ G418). Following induction of ES-cell differentiation, neural-restricted cells (blue) can be isolated from non-neuronal cells (mesoderm in pink and endoderm in grey) by cell sorting or drug selection as appropriate* **(B)**. *Neural precursor cells can then be expanded under selective pressure (that is, in the presence of G418).* **(C)** *shows a population of neuronal cells derived from ES cells following selection for expression of Sox2. Double labelling with propidium iodide (orange) shows that all cells possess the neuronal marker Tuj1 (β-tubulin III; green). Scale bar, 20 μm.*

Selection of neural precursors from ES-cell populations

A possible solution to the difficulties encountered when transplanting ES cell progeny would be to preselect the population for transfer, in order to eliminate irrelevant cells. Where suitably specific cell-surface markers are available, as in the haematopoietic system, immunoselection is an option. An alternative route involves engineering a lineage-selection marker into the ES cells by genetic manipulation, either homologous recombination or transgene addition. The selection marker should be coupled to a promoter that is active solely in the cell population of interest. Following induction of differentiation, cells that express the lineage-restricted gene will also express the marker and can be purified by cell sorting or drug selection (Fig. 1). Genes for transcription factors are often expressed in suitable lineage-restricted or cell type-restricted patterns. Thus the high-mobility group (HMG)-box transcription factors, SOX1, SOX2 and SOX3, are found throughout the

neuroectoderm of the early embryo but are absent from other developing lineages[40]. Selection for expression of G418 resistance linked to *Sox2* expression has been successfully employed to enrich for neural precursors during ES-cell differentiation[34]. This type of approach is likely to prove invaluable for producing cultures of ES-cell-derived neural precursors, which can then be used for detailed characterization and comparison with foetal- and adult-derived neural stem cells. The isolation of homogeneous, developmentally labile, cultures of primitive neural cells will also allow the examination of their responsiveness to candidate inducers of neuronal subtype specification, such as sonic hedgehog and fibroblast growth factors[41]. A further selection marker could even be incorporated in order to allow the purification of appropriately induced cells, such as dopaminergic-neurone precursors. Combining induction and selection might be a route to generating defined populations for transplantation.

Can human ES cells be grown in culture?

Until recently, ES or EG cells that were capable of multi-lineage differentiation had only been reported in specific strains of mice[42]. However, two recent reports present evidence for the tantalizing possibility that ES-like cells might be isolated from humans[18,19]. Thomson *et al.* used surplus embryos donated by individuals undergoing infertility treatment. These embryos were allowed to develop to the blastocyst stage and were then treated in essentially the same manner as for derivation of mouse ES cells. In this way, five diploid cell lines were obtained from 14 blastocysts. Each of these cell lines produced teratomas when injected into immunocompromised mice (Fig. 2). In the second report, Shamblott and colleagues described cultures derived from foetal gonadal tissue obtained after elective termination of pregnancy. Immunohistological analyses revealed an assortment of differentiated cell morphologies in these cultures.

These reports are very exciting and suggest that our experience with mouse ES cells might be transferred directly to humans. However, caution is still required at this early stage of our understanding in this new area. It is not certain that human cells derived by either the blastocyst or germ-cell route, will show equivalent properties to mouse ES cells, as highlighted in Box 3. Clonal analysis has not yet been performed, which is a pre-requisite for a formal conclusion of pluripotent stem-cell identity. Furthermore, the definitive test of a rodent ES cell is its ability to contribute to chimaeras, a criterion that is not applicable to human cells for practical and ethical reasons. In this regard it might be more appropriate to describe these cells as human pluripotent stem cells (HPCs) rather than human ES cells. The ability to expand the stem-cell population is crucial, as many millions of cells will be needed for neural transplants. The proliferative capacity of these HPC cultures is far from clear, but it appears that they are rather more difficult to expand than mouse ES cells. It is also uncertain as to whether these human cells use the same intracellular signalling pathways as mouse ES cells[28] in order to sustain the self-renewal cycle. The ability of the human cells to produce foetal cell types *in vitro* also has to be confirmed. Surprisingly, only extra-embryonic cell types were detected when differentiation of the blastocyst-derived cells was induced *in vitro*[18]. This could simply be because appropriate permissive or inductive conditions have not been identified. However, the contrast with the description of multi-lineage differentiation in PGC-derived cultures[19] is intriguing and raises the possibility that human stem cells derived from the blastocysts or germ cells might not be equivalent. Finally, the possibility of transformation will always be a concern with cultured cells, and screens will have to be developed to detect and eliminate mutations.

Neural precursor cells can be isolated from human foetal tissue

The human blastocyst-derived cells generate neuroepithelial structures in teratomas (Fig. 2). If this can be reproduced *in vitro*, it should be possible to isolate neural precursors, as described above for mouse ES cells, which in turn might circumvent the need for foetal CNS tissue. However, as indicated in the previous section, it is not yet clear how rapidly the human pluripotent cells can be expanded in culture, how stable they will be over repeated passages or how pure the neural cells derived from them will be. Thus, it is important to complement ES-cell work with other approaches used to generate neural tissue in culture. During development, stem cells in different organs become increasingly restricted with regard to their phenotypic potential. The neuroepithelium generates all of the CNS and is thought to contain a population of founder neural stem cells. A more-liberal approach has been taken with regard to defining neural stem cells when compared to ES cells, in part, because this field is very new and many aspects remain to be fully characterized[43]. However, neural stem cells should at least be multipotent and capable of extended self-renewal, characteristics that can only be revealed using clonal analysis where the progeny of single cells can be assessed. This has been demonstrated for both epidermal-growth-factor (EGF)-responsive and FGF2-responsive cells derived from various regions of the embryonic or adult rodent CNS, and grown as either cell aggregates (neurospheres) or monolayer cultures[44–49]. In a very recent development, cloned single neural stem cells that were derived from the adult mouse brain have been shown to differentiate into blood cells following transplantation into irradiated mice[50]. This exciting finding suggests that neural stem cells might have a wider potential for differentiation than was previously assumed[51], but has yet to be confirmed by other groups.

The extrapolation of these rodent studies on neural stem cells to the human remains in its early stages. Cells isolated from the 5–12-week-old human foetal CNS have been grown attached to a substrate in the presence of FGF2, and have been found to generate neurones, astrocytes and oligodendrocytes[52]. Similar EGF-responsive cells could be isolated from the mesencephalon of older fetuses (>13 weeks), but not younger ones, and grown as neurospheres in serum-free defined medium[53]. Other groups have had similar problems in inducing the division of human neural precursors from young tissues (6–8 weeks) with EGF alone, and needed to use both 5% horse serum in combination with insulin-like growth factor 1 to expand the cells as neurospheres[54]. This lack of EGF responsiveness might be due to the late development of EGF receptors[55]. Furthermore, it is clear that mouse striatal precursors can be 'primed' with FGF2, after which they will then respond to EGF (Ref. 56), suggesting that FGF2-responsive cells give rise to the EGF-responsive cells. Human FGF2-responsive precursors

grown for short periods of time as neurospheres have been shown to express the polysialylated form of neural cell-adhesion molecule and to generate a small number of oligodendrocytes that can respond to thyroid hormone[57]. As all of these studies have used population rather than clonal analyses, it is not possible to assess the multi-potentiality of individual cells. However, in most studies, neurones, astrocytes and oligodendrocytes (in very small numbers) were found within the cultures following the withdrawal of the mitogen and the exposure to a suitable substratum. In this article, the favoured terminology for cells within these types of cultures, where cell division is clearly taking place but the status of individual cells cannot readily be determined, is human neural precursor cells (HNPCs).

HNPCs can be cloned, and integrate into the developing and adult CNS

Flax *et al.*[21] attempted to address whether HNPCs were in fact multipotent by taking individual FGF2-responsive cells, which were derived from the embryonic CNS, and generating a clonal line. In one example, such a line could be expanded *in vitro* and showed apparent multipotency, on the basis of results obtained using immunocytochemical markers. Parallel clones were generated from similar human cells immortalized by *Myc* according to protocols used previously for rodent tissues[58]. All clones appeared to be similar in culture while only some could engraft into the developing brain. This is the first evidence that a single human cell is capable of expansion *in vitro* and subsequently capable of producing neurones, astrocytes and oligodendrocytes, thus fulfilling the main criteria that define a neural stem cell. However, the percentage of non-immortalized clones that gave rise to all three phenotypes was not presented. Furthermore, no information was given regarding the length of time *in vitro* that the clones had been expanded prior to differentiation or grafting, or how this related to the numbers of neurones and glia generated. This is an important issue, as it is possible that the original stem cell will undergo both symmetrical and asymmetrical divisions *in vitro*, leading to a heterogeneous pool of both committed progenitors and new stem cells[59].

Flax *et al.* also showed that following grafting into the postnatal day 0 (P0) mouse brain, human precursor cells expanded for short periods of time in culture could integrate into both developing forebrain and cerebellar structures, even replacing granule cells lost in the neurone-deficient cerebellum of the Meander tail mutant[21]. Remarkable integration was also shown to occur when similar HNPCs, expanded for short periods of time and generated from bulk culture, were transplanted into the developing embryo. Human cells mixed almost seamlessly with the rodent ones forming a 'chimaeric' brain[24]. In this study, human oligodendrocytes were also found in close proximity to rodent axons and might have been been able to myelinate under these transplant conditions. Neurones were also found (Fig. 3), although their specific neurochemical phenotypes were not reported.

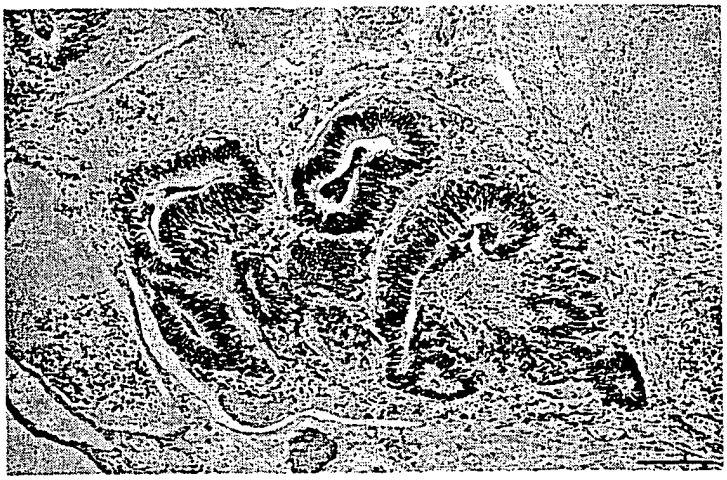

Fig. 2. Teratomas formed by human blastocyst-derived stem-cell lines. Human cells after four to five months of culture (passages 14–16) from about 50% confluent six-well plates were injected into the rear leg muscles of four-week-old male immunodeficient mice (two or more mice per cell line). Seven to eight weeks after injection, the resulting teratomas were examined histologically. The figure shows rosettes of neural epithelium. Scale bar, 200 μm. Courtesy of James Thomson.

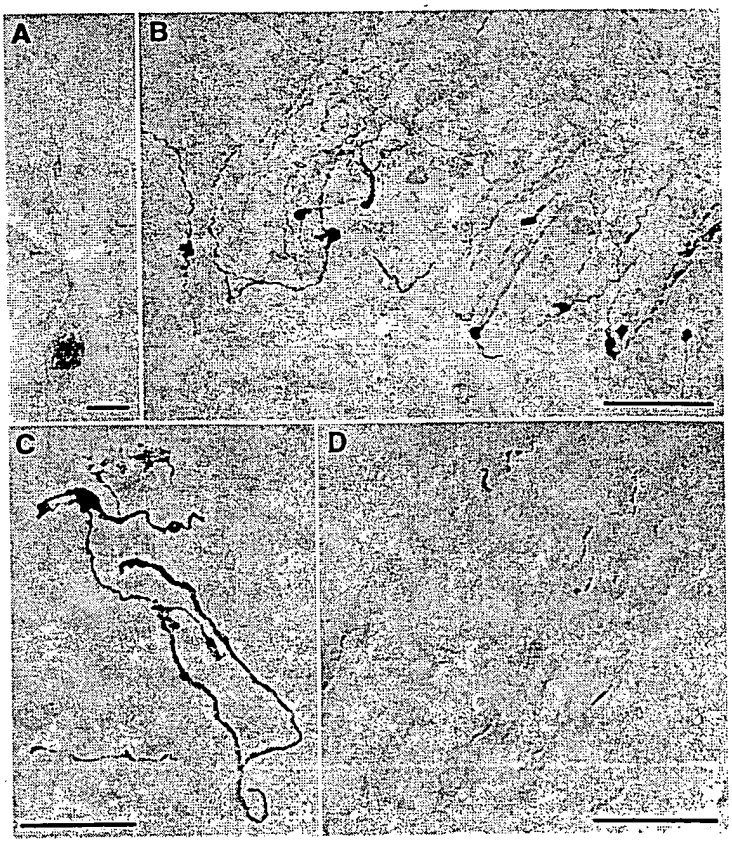

Fig. 3. Human neurones incorporated into the developing rat brain. (A) An individual neurone, hybridized with the human-specific alu-probe repeat element (black) and double labelled with a human-specific antibody to neurofilament (hNF-M; brown). Freshly dissociated human precursor cells were injected into embryonic-day 17 (E17) hosts that were sacrificed after 30 days. (B) and (C) Immunohistochemical detection of β-galactosidase-positive human cells with neuronal morphologies in (B) tectum and (C) hypothalamus of two-week-old recipients. Donor cells were grown for four weeks in defined medium containing 10 ng/ml fibroblast growth factor 2 and were transduced with an adenovirus carrying the lacZ gene and transplanted into E17 recipients. (D) Axons stained for hNF-M at the transition of corpus callosum and cortex in humans, 51 days after intraventricular transplantation of seven-week-old epidermal growth factor-generated neural spheres. Scale bars, 10 μm in (A), 100 μm in (B), 50 μm in (C) and 20 μm in (D). Reproduced, with permission, from Ref. 24.

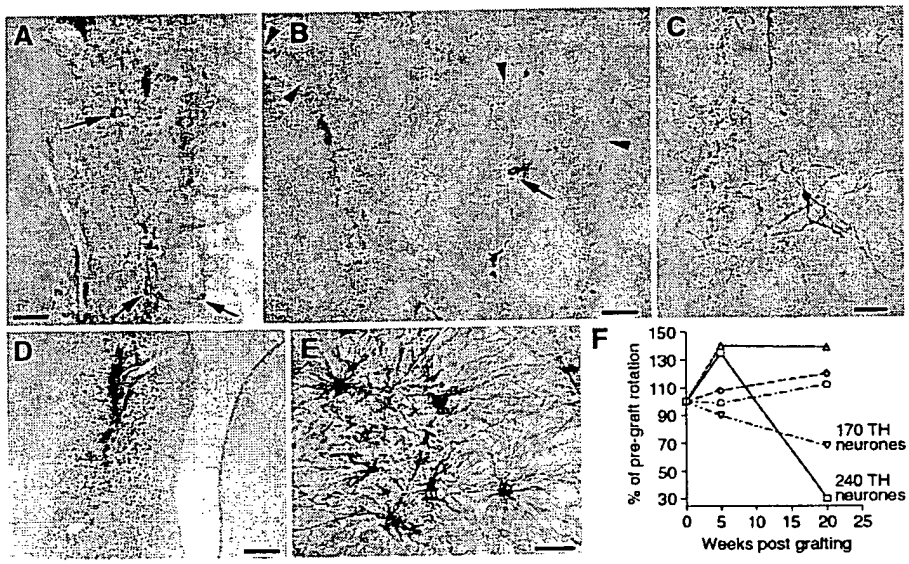

Fig. 4. *Human neural precursors integrate into the adult rat CNS. Precursor cells isolated from a 22-week-old cortex were expanded* in vitro *for 21 days with a combination of epidermal growth factor and fibroblast growth factor 2, and then injected into the striatum of rats with lesions of the dopaminergic system.* (A) *Most grafts thinned over time and by 20 weeks only remnants of graft mass remained. Within this remnant, a number of cells stained for the neuronal marker human tau can be seen (arrows). The arrow indicates a neuronal cell body. This antibody does not react with rat neural tissue.* (B) *High-power photomicrograph showing human tau staining within fibres coursing through the striatum (arrowheads).* (C) *Staining for tyrosine hydroxylase (TH), which labels dopaminergic neurones, reveals a number of positive cells (black) in two animals that have extensive ramifications into the striatum. These cells were found around the graft site in close proximity to the human tau-stained neurones and were not seen in any of the sham-grafted animals.* (D) *Many more cells have migrated from the graft core and matured into astrocytes, labelled here with human glial fibrillary acidic protein (GFAP), a specific marker for human astrocytes. This human GFAP antibody will label highly reactive rodent astrocytes following a lesion, but not 20 weeks after the lesion. All sham-grafted animals were negative for human GFAP staining at 20 weeks.* (E) *High-power photomicrograph of* (D) *that shows human astrocyte morphology.* (F) *Graph showing rotation scores. Following a unilateral 6-hydroxydopamine lesion, rats showed the characteristic pattern of rotation towards the lesioned side at the rate of 12.6±1.74 complete turns per minute. Following transplantation only two animals showed differences in rotation at 20 weeks post grafting (expressed as percent of pre-graft rotation) and these were the only two animals with any TH-positive cells within the striatum (the number of cells is shown beside final data points on graph). Scale bars, 150 μm in* (A), *100 μm in* (B) *and* (C), *250 μm in* (D), *and 100 μm in* (E). Adapted, with permission, from Ref. 60.

These studies show that HNPCs can differentiate in response to local developmental cues in the embryonic or neonatal rodent, suggesting a conservation of such signals across species and an inherent cellular plasticity. But what happens when these cells are transplanted into the adult rat brain, a situation perhaps more analogous to that of clinical transplantation? HNPCs derived from the human embryonic cortex and expanded in culture for between two and four weeks (passaged every week) with EGF and FGF2 can survive transplantation into the striatum of adult rats with dopaminergic-neurone lesions, and develop into neurones and astrocytes[6]. Some of these human neurones show extensive axonal branching into the host tissue (Fig. 4A,B). However, unlike transplants of primary, non-expanded tissue, there was extensive migration away from the graft site and into the host brain. Many of these migrating cells were astrocytes (Fig. 4D,E). In some animals, a small percentage of neurones expressed the gene for tyrosine hydroxylase and were able to reverse behavioural deficits associated with the lesion (Fig. 4C,F). This suggests that there could be signals within the adult striatum that are able to direct HNPCs into a dopaminergic phenotype. Similar studies with genetically modified non-passaged FGF2-responsive HNPCs, which were derived from the cortex and grown as monolayers, have shown that surviving cells can be found within a graft mass, but only when injected at high density (>1 million cells)[61,62]. Thus, in order to establish neuronal-precursor grafts that retain cell mass it might be necessary to transplant larger cell numbers, although this might not relate directly to fibre outgrowth from the transplant. Alternatively, HNPCs can be pre-differentiated prior to transplantation. This has been shown to lead to the formation of small graft masses of HNPCs following transplantation into the adult lesioned striatum, although no staining for dopaminergic neurones was carried out in this very recent study[63].

Although these results are encouraging, it will be crucial to obtain larger numbers of dopaminergic neurones before clinical transplantation programmes for Parkinson's disease could be considered using precursor cells. The default neuronal phenotype from expanded populations of precursor cells *in vitro* appears to be GABAergic. However, transcription factors such as *Nurr1* and *Ptx3*, which could regulate dopaminergic phenotype[64,65], are currently being investigated. In addition, other factors have been found that induce dopaminergic neurones in the developing mesencephalon, such as sonic hedgehog and FGF8 (Ref. 41). It is possible that combinations of these transcription and growth factors will, in future studies, be able to induce a dopaminergic phenotype in neurones that differentiate from human precursors, which in turn could be used in transplantation programmes.

HNPCs can be propagated continually *in vitro*

Although these recent reports on cloning and transplantation are of enormous interest, there have been few systematic studies where the real growth potential of human neural precursors has been assessed. The EGF-responsive mouse cells that have been isolated from the developing striatum show exponential growth for long periods of time in culture as neurospheres[48], although these mainly give rise to astrocytes upon differentiation or transplantation[66,67]. However, under identical culture conditions, rat cells consistently stopped dividing between four and seven weeks of growth[68]. When growth curves were established for HNPCs grown as neurospheres, a slow down in cell division with increasing passages was also noticed. On the basis of observations that clusters of cells divide better than isolated cells in culture[69], and cell density can increase precursor cell survival[70], a novel method of passaging has recently been developed where the growing neurospheres are sectioned into quarters rather than mechanically dissociated[22,59]. This maintains cell–cell contact and allows the exponential growth (over 1 million-fold increase

in cell number in 150 days) of HNPCs without any special modifications to the medium. Either FGF2 or EGF alone can maintain these cultures. Remarkably, and in stark contrast to the rodent tissues, even after extended growth these neurospheres are karyotypically normal and able to give rise to over 40% neurones, providing cell–cell contact is also maintained during the differentiation period. Interestingly, although small numbers of oligodendrocytes were found in this study, and by others[57] at early passages, very few can be seen at late passages in our cultures. This suggests that either the correct environment for oligodendrocyte differentiation has not been established, or that the dividing cells are able to produce only neurones and astrocytes, and by definition should be considered bi-potent progenitors. Canine and rodent studies have shown that conditioned medium from the cell line, B104, can support the growth of 'oligospheres'[71,72]. However, the effects of these conditioned medium factors on human cells have not yet been established.

Other groups have now reported on the long-term growth of human neural stem cells. One study has confirmed other observations and was unable to obtain significant expansion of these cells for long periods using normal media containing EGF and FGF2. However, the addition of LIF to the culture medium was able to overcome this growth restriction and allow a significant amount of expansion to occur[23]. Interestingly, LIF, which acts through the gp130 signal transducing subunit, is also required for the continual growth of ES cells and maintains these cultures in a proliferative state by preventing cellular differentiation[7,73]. Another very recent report suggests that while EGF or FGF2 alone are unable to propagate human neural stem cells continually, a combination of both can maintain such cells in a mitotic state for extensive periods of time. Interestingly, this report used traditional passaging methods and did not require LIF (Ref. 63). However, the percentage of neurones differentiating from such cultures was significantly lower than that seen for the sectioning method or LIF-supplemented cultures.

It should be stressed that the majority of studies using human foetal tissue to generate neural stem cells have used forebrain or cortex as the starting region. It is possible that other brain regions could give rise to different proportions of neurones and glia, and divide for different amounts of time *in vitro*. These issues are currently under investigation. However, it is clear that cortically derived neural precursor cells provide a readily available source of expandable human neural tissue.

Neurogenesis continues in defined regions of adult human CNS

In addition to generating neurones from foetal stem cells, there are now considerable data showing that the subventricular zone and dentate gyrus of a wide range of mammalian species continue to generate neurones well into adulthood[74,75]. These newly generated cells might be specifically located within the ependymal layer, at least in rodents[76]. Similar cells had previously been difficult to detect in primate brain tissue[77] until a recent report suggested that neurogenesis did continue within the dentate gyrus of adult marmoset monkeys[78]. *In vitro* studies have suggested that explants of the hippocampus taken from epilepsy patients contain cells that divide and generate neurones in culture[79]. It could also be possible to expand these human adult precursor cells with mitogens, which would provide another source of human neural tissue, although to date this has not been reported. Although these are interesting *in vitro* data, it is not clear whether this process actually occurs *in situ* within the normal human brain. By studying the post-mortem brains of patients who had received large doses of BrdU while alive, Eriksson *et al.*[80] have recently shown that the adult human hippocampus does contain cells within the dentate gyrus that continue to divide into adulthood. However, the multipotency of these cells is not known at present. They could represent a population of resident stem cells, or a specialized neuronal progenitor that continues to divide throughout life. Either way, this suggests that neurogenesis continues in at least some regions of the adult human brain. Thus, future therapeutic strategies might also be aimed at generating HNPC cultures from adult tissues (ideally from the same patient that requires the therapy), or stimulating these cells to divide *in situ*, migrate and repair local neuronal damage.

Human pluripotent and neural precursor cells: implications for cell and gene therapy

After decades of genetic and cellular research using model organisms, there is now the opportunity to study human neural tissues directly. This is important for a number of reasons. First, there are significant genetic differences between rodents and humans, reflected by the fact that many antibodies, probes and drugs are species-specific. Furthermore, it is now clear that human neural precursor cells are very different from their rodent counterparts and need to be assessed independently: it is not possible to extrapolate from mouse to man directly. Thus, results with human cells will be more directly relevant to medical applications. Second, within the next few years the human genome project will provide a complete list of human genes. Using human tissues, the regulation and cellular function of such genes can be assessed directly. This is particularly relevant when combined with homologous recombination and ES-cell technology where gene alterations can be made in specific locations and all resulting progeny from these stem cells (including neurones) will subsequently carry this modification. In this way, specific genes could be deleted, modified or added in order to assess their function, mimic the pathology of common inherited disorders, or express therapeutic proteins, such as neurotrophic factors. Finally, ES and neural precursor cells could potentially be used in the new fields of cell and gene therapy[81–83], which will require large amounts of human tissue. Genetic manipulation could also provide a means of customizing the stem cells for each patient in order to minimize concerns of immunological rejection. For example, genome-engineering technologies[84] could be used to mobilize major histocompatibility loci. Minor loci would always remain with such an approach, however, and might present a problem. The ultimate solution, therefore, would be to recreate stem cells from the patient's own tissue[20]. In principle this should be achievable via nuclear transplantation into an enucleated oocyte[85] followed by development in culture to the blastocyst stage. Stem cells could then be isolated, expanded and selected prior to differentiation and autologous transplantation (Fig. 5). The challenge of translating our still rudimentary understanding of mouse embryology, ES-cell biology, neural stem-cell biology and nuclear reprogramming into a system for human

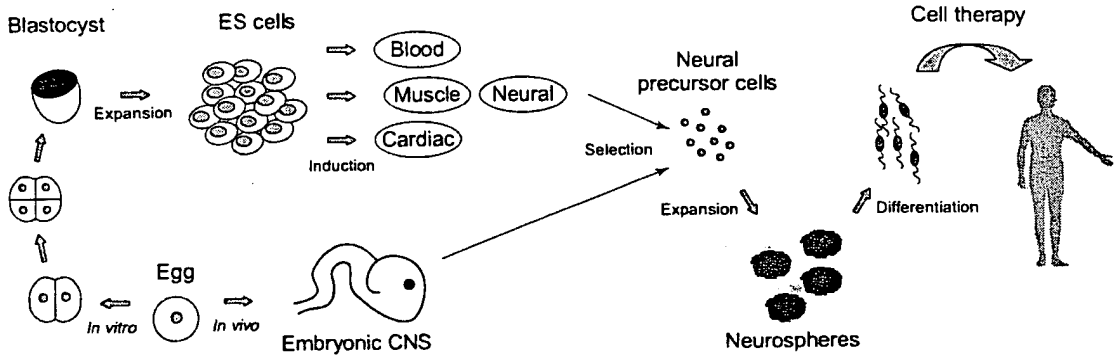

Fig. 5. *Stem-cell therapy for CNS disorders. Two alternative routes for generating neural tissue are described. Embryonic stem (ES) cells could be derived from fertilized eggs (which might also have been subject to host nuclear transfer). From these ES cells, neural precursors could be selected from other tissue types, using the methods outlined in Fig. 1. Alternatively, neural precursor cells could be isolated from post-mortem embryonic human neural tissues. In both cases the neural precursors can then be expanded in culture, differentiated into specific neural phenotypes (neurones, astrocytes or oligodendrocytes) and then transplanted into patients with neurodegenerative diseases. Nuclear transfer of host nuclei could be performed into the oocytes while genetic modification could be performed at stem-cell stages. Cells could be expanded at either the ES or neural precursor-cell stages, providing enormous leverage with regard to absolute cell growth and the generation of clinically useful amounts of tissue.*

cell therapy remains considerable. However, the principle avenues of investigation are becoming clear and the prize in prospect is great.

Note added in proof

A recent publication has shown that HNPCs that have been expanded 10 million-fold migrate and differentiate appropriately when transplanted into neurogenic regions of the adult rat brain[86]. These data further support the use of such cells in both experimental and clinical transplantation programmes.

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BMP4 initiates human embryonic stem cell differentiation to trophoblast

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The excitement and controversy surrounding the potential role of human embryonic stem (ES)[1,2] cells in transplantation therapy have often overshadowed their potentially more important use as a basic research tool for understanding the development and function of human tissues. Human ES cells can proliferate without a known limit and can form advanced derivatives of all three embryonic germ layers. What is less widely appreciated is that human ES cells can also form the extra-embryonic tissues that differentiate from the embryo before gastrulation. The use of human ES cells to derive early human trophoblast is particularly valuable, because it is difficult to obtain from other sources and is significantly different from mouse trophoblast. Here we show that bone morphogenetic protein 4 (BMP4), a member of the transforming growth factor-β (TGF-β) superfamily, induces the differentiation of human ES cells to trophoblast. DNA microarray, RT-PCR, and immunoassay analyses demonstrate that the differentiated cells express a range of trophoblast markers and secrete placental hormones. When plated at low density, the BMP4-treated cells form syncytia that express chorionic gonadotrophin (CG). These results underscore fundamental differences between human and mouse ES cells, which differentiate poorly, if at all, to trophoblast[3]. Human ES cells thus provide a tool for studying the differentiation and function of early human trophoblast and could provide a new understanding of some of the earliest differentiation events of human postimplantation development.

Human ES cell lines H1, H7, H9, and H14 (ref. 1) were cultured on Matrigel-coated plastic plates in conditioned medium (CM) from mouse embryonic fibroblasts and supplemented with basic fibroblast growth factor (bFGF) at 4 ng/ml to maintain their undifferentiated proliferation[4]. Recombinant human BMP4, added at concentrations of 1, 10, and 100 ng/ml to ES cells cultured in CM in the continuous presence of bFGF, induced a dose-dependent morphological change of the cells. Over a period of days, a synchronous wave of differentiation occurred, characterized by flattened, enlarged cells with reduced proliferation (Fig. 1A, B; a time-lapse film is available online (see URLs in Experimental Protocol)). The morphological changes became obvious on day 2 for BMP4 at 100 ng/ml, days 3–4 for 10 ng/ml, and days 4–5 for 1 ng/ml. BMP family members, such as BMP2 (300 ng/ml), BMP7 (300 ng/ml), and growth and differentiation factor-5 (GDF5) (30 ng/ml), induced similar morphological changes. However, other TGF-β superfamily members, such as TGF-β1 (0.01–0.1 ng/ml) and activin A (0.1–5 ng/ml), did not induce any noticeable morphological changes. The addition of inhibitors of BMP signaling, such as the soluble BMP receptor (human BMPR-IB/Fc chimera; 30 ng/ml) or the BMP-antagonizing protein noggin (300 ng/ml), blocked the morphological changes induced by the BMPs. When detached and maintained in suspension culture, the BMP4-induced cells formed vesicles (see Supplementary Fig. 1 online). ES cells cultured in unconditioned medium with or without bFGF also differentiated, but the differentiation was more asynchronous, resulting in a morphologically mixed population of cells, and this differentiation could not be blocked by the soluble BMP receptor or by noggin. BMP4 accelerated the differentiation observed in the absence of bFGF or CM. No morphological change was observed when ES cells were treated with the soluble BMP receptor or noggin alone (data not shown).

In contrast to the mononuclear cells that formed after BMP4 treatment of ES cell colonies, syncytial cells were present among individualized BMP4-treated ES cells plated at low density. For example, in one experiment in which we plated H1 cells as single cells at low density and treated them with 100 ng/ml BMP4, we observed 44 syncytia among 622 cells after two weeks of treatment. These syncytial cells contained different numbers of nuclei (from 2 to 100) and were positive for CG-β on immunostaining (Fig. 1C, D). Time-lapse movies demonstrated that these multinucleated cells formed by fusion and not by endoduplication. Injection of rhodamine-dextran confirmed that the multiple nuclei shared a continuous cytoplasm (see Supplementary Fig. 2; a time-lapse film is available online (see URLs)).

We used cDNA microarrays to analyze genes differentially expressed in the BMP4-treated and the untreated, undifferentiated H1 cells, both cultured in the continuous presence of CM and bFGF (Fig. 2A). Of 43,000 cDNA clones examined on the arrays, a cluster of only 19 clones, representing 14 genes, was strongly upregulated at all the time points examined. Of these, 11 were previously described as genes related to the development of trophoblast or placenta (Fig. 2B). Many of the genes encode transcription factors, such as transcription factor AP-2 (TFAP2)[5], msh homeobox homolog 2 (MSX2), and suppressor of cytokine signaling 3 (SSI3) (ref. 6), GATA binding proteins 2 and 3 (GATA2 and GATA3)[7], SSI3 (ref. 8), and hairy/enhancer-of-split related with YRPW motif 1 (*HEY1*)[9]. By day 7 of BMP4 treatment, there was a dramatic increase of mRNA expression of many genes expressed in trophoblast or placenta, such as those encoding CG-α and CG-β subunits, luteinizing hormone-β, and placental growth factor[10,11] (Fig. 2C). Using RT-PCR, we also observed enhanced expression of trophoblast markers, including CG-β, glial cells missing-1 (GCM1)[12], the non-classical HLA class I molecule HLA-G1, and CD9 (ref. 13) (see Supplementary Fig. 3 online). All of the top ten upregulated clones (representing eight genes) in the microarray of the day 7 BMP4-treated cells, except for one cDNA that was not studied, encode proteins or peptides previously described as being expressed by the trophoblast. These include CG-α, CG-β[10], endothelial PAS domain protein 1 (ref. 14), insulin-like growth factor–binding protein 3 (ref. 15), iodothyronine deiodinase type III (ref. 16), GATA2 (ref. 7), and glutamyl aminopeptidase[17] (see Supplementary Table 1 online). Some genes whose homologs are known to be important for mouse trophoblast, such as those encoding cytokeratin 7, human achaete scute homolog 2 (HASH2), estrogen-related receptor-β (ERR-β), and hepatocyte growth factor receptor (MET)[12], were not elevated as compared with ES cells (see Supplementary Tables 2.1 and 2.2 online). However, RT-PCR demonstrated that these genes were expressed both by the undiffer-

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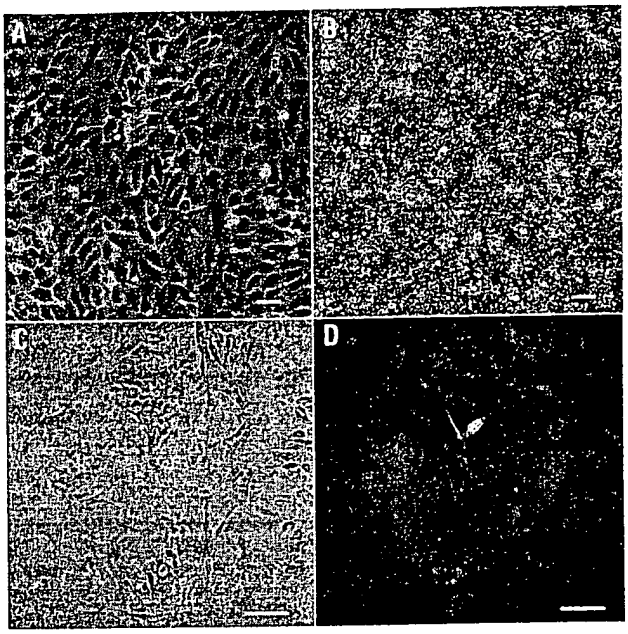

Figure 1. Morphological changes of BMP4-treated H1 cells. (A,B) H1 cells (cultured in CM with bFGF) were treated with (A) or without (B) 100 ng/ml BMP4 for seven days. (C) A syncytial cell formed after two weeks of treatment of individualized ES cells by BMP4. (D) Immunofluorescence for CG-β (green) and Hoechst 33342 fluorescence for the nuclei (blue). Bars, 25 μm.

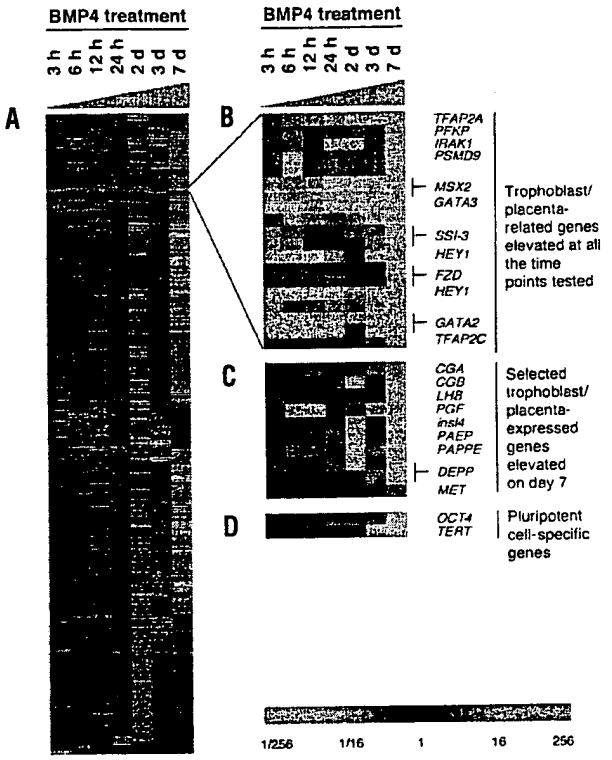

Figure 2. Microarray analysis of BMP4-treated H1 cells. H1 cells (cultured in CM with bFGF) were treated in pairs with or without 100 ng/ml BMP4. Each pair of cells was harvested at various times up to seven days. (A) Microarray of 3337 cDNA clones that showed at least threefold changes in the gene expression between a pair of BMP4-treated and untreated samples at one or more times during the treatment. Gray indicates missing or excluded data. (B–D) Expanded views of characteristic gene expression patterns. (Refer to Supplementary Tables 2.1 and 2.2 online for the raw data and gene search.)

entiated ES cells and by the BMP4-treated, differentiated cells (see Supplementary Fig. 3 online). By day 7 of BMP4 treatment, transcripts of genes highly expressed in pluripotent cells, such as those encoding POU domain, class 5, transcription factor 1 (POU5F1, also known as OCT4)[18] and telomerase reverse transcriptase (TERT)[19], were significantly decreased in both by microarray (Fig. 2D) and RT-PCR analysis (see Supplementary Fig. 3 online). Also at day 7, expression of genes characteristic of endoderm (for example, those for α-fetoprotein, hepatocyte nuclear factor, and PDX1), mesoderm (for example, those for brachyury, eomes, and chordin), and ectoderm differentiation (for example, those for cellular retinoic acid binding protein-1, sex-determining region box-2, and nestin) was not significantly elevated in the BMP4-treated cells relative to controls (see Supplementary Tables 2.1 and 2.2 online).

Because some non-trophoblast-derived tumor cell lines express trophoblast markers, we compared expression profiles of one such tumor cell line (HeLa cells[20]) with BMP4-treated human ES cells. Microarray comparison of these two kinds of cells showed that there is little similarity in their gene expression profiles. HeLa cells did express CG-α at about half the level seen in the BMP-treated ES cells (see Supplementary Fig. 4 online), but none of the other trophoblast markers that dominated the top upregulated genes in the BMP4-treated cells was expressed at high enough levels to be included in the cluster analysis for HeLa cells (a threefold ratio is required for inclusion). On the other hand, tumor markers such as the MAGE and GAGE families of tumor-associated antigens were highly expressed in HeLa cells but not in the BMP4-treated ES cells (see Supplementary Fig. 4 online).

To seek additional confirmation of trophoblast differentiation of BMP4-treated ES cells, we measured the amount of the placental hormones CG, estradiol, and progesterone secreted into the medium. H1 cells treated with BMP4 showed markedly higher concentrations of each hormone than did either undifferentiated cells or cells differentiated in unconditioned medium (Fig. 3). Fluorescence-activated cell sorting analysis of permeabilized BMP4-differentiated H1 cells labeled by an antibody to the CG-β subunit demonstrated a surprisingly uniform shift of the population to CG-β expression (Fig. 4).

To determine whether BMP4 treatment of ES cells provides an instructive signal for the differentiation of trophoblast or whether it provides a selective signal for trophoblast cells that had already committed to differentiate, we followed the fate of individual ES cells during BMP treatment by time-lapse microscopy for three days (see Supplementary Table 3 online). Over the three-day period, 119 BMP4-treated ES cells gave rise to 322 final cells, all with a flattened, differentiated morphology, and during those three days, 34 cells died and detached. In the control culture (no BMP4 treatment) during the same period, 137 ES cells gave rise to 330 cells, all with a typical ES cell morphology, and 59 cells died and detached. These results are most consistent with the model in which BMP4 has an instructive effect on trophoblast differentiation.

The first differentiation event in mammalian embryos is the formation of the trophectoderm, the outer epithelial layer of the blastocyst. The trophectoderm is crucial for implantation of the embryo and gives rise to specialized populations of trophoblast cells in the definitive placenta[12,21]. When formed into chimeras with intact preimplantation embryos, mouse ES cells rarely contribute to the trophoblast, and the manipulation of external culture condi-

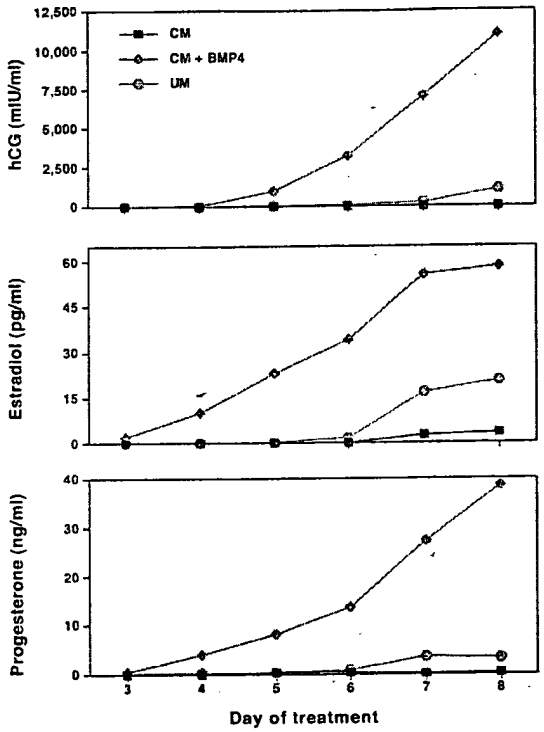

Figure 3. Immunoassays of placental hormones. Culture medium conditioned by H1 cells cultured in CM (gray square), CM + BMP4 (100 ng/ml) (red diamond), or unconditioned medium (green circle) (all in the continuous presence of bFGF) were collected at the indicated times and subjected to immunoassay for human chorionic gonadotropin (hCG), estradiol, and progesterone.

tions has, to date, failed to direct mouse ES cells to trophoblast[3]. The failure to form trophoblast is consistent with the idea that mouse ES cells are developmentally similar to primitive ectoderm, which forms after delamination of the primitive endoderm from the inner cell mass and which no longer contributes to the trophoblast[22]. However, the forced downregulation of Oct4, which is essential for ES cell pluripotency, results in a de-differentiation of mouse ES cells to trophoblast[23]. The ability of human ES cells to form trophoblasts during spontaneous, mixed differentiation in the absence of CM and bFGF[1,2] or after BMP4 induction suggests a basic difference between the developmental potential of mouse and of human ES cells. This difference is also suggested by the behavior of embryonal carcinoma cells, which are the malignant counterpart of ES cells and the stem cells of teratocarcinomas. Human teratocarcinomas often contain a trophoblast component, but mouse teratocarcinomas do not[24,25]. Mouse trophoblast stem cells have been derived from both the trophectoderm and the later extraembryonic ectoderm[26]. Mouse trophoblast stem cells can contribute to multiple trophoblast populations in chimeras and depend, in part, on fibroblast growth factor signaling for their undifferentiated propagation[26]. The human equivalent to trophoblast stem cells has not yet been derived, and it is likely that different growth factors will be required for their propagation[27]. Although, in our current studies, BMP4 efficiently induced differentiation of human ES cells to trophoblast, these trophoblast cells propagated poorly, even in the continued presence of bFGF and fibroblast feeder layers (data not shown), suggesting that additional growth factors are required for their long-term proliferation.

Human ES cells offer an important new window into early human developmental events, and the present report underlines both the power and an inherent weakness of this new model. A major strength of human ES cells is that they give access to early human cell types that were previously almost unobtainable. A major weakness is that ethical considerations will make it extremely difficult to confirm that *in vitro* results with these early cells have *in vivo* significance. We demonstrated here that BMP4 can induce human ES cell differentiation to trophoblast *in vitro;* however, a direct role of BMPs in early trophoblast differentiation *in vivo* has not, to our knowledge, been demonstrated in any mammal. Transcripts of various BMP receptors are present in morula- and blastocyst-stage mouse embryos, and transcripts of BMPs are present in the maternal tissues surrounding the embryos[28]. It has also been reported that BMP receptors are present on human ES cells[29]. The challenge for the future will be to determine whether BMP signals have a role in human trophoblast differentiation *in vivo* and to identify what signals sustain the proliferation of early trophoblast cells and direct them to become the multiple trophoblast populations of the definitive human placenta.

Experimental protocol

Cell culture, treatment, and syncytium analysis. Human ES cell lines H1, H7, H9, and H14 were cultured as described[1,4]. Briefly, they were plated as colonies and cultured in mouse embryonic fibroblast CM supplemented with 4 ng/ml human bFGF (Life Technologies, Rockville, MD) in six-well plates precoated with Matrigel (Becton Dickinson Labware, Bedford, MA). Protein factors were added directly to the culture in the continued presence of CM and bFGF, unless otherwise noted. Cell morphology was photographed at designated times or by time-lapse photography. For syncytium formation and analysis, H1 cells were individualized by treatment with trypsin/EDTA solution (Life Technologies) for 15 minutes at 37°C and plated at low density; some plates were then treated daily with 100 ng/ml BMP4 (R&D Systems, Minneapolis, MN, also the source for other recombinant proteins tested). Some of the individualized BMP4-treated H1 cells formed syncytial cells within two weeks of the treatment. These cells were treated with the Golgi blocker brefeldin A (Sigma, St. Louis, MO) at 1.25 μg/ml for 4 h at 37°C, fixed with 2% paraformaldehyde for 10 min, and immunos-

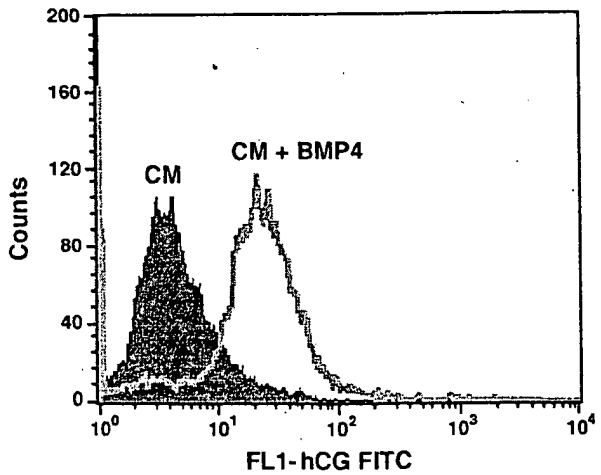

Figure 4. Fluorescence-activated cell sorting analysis for CG-β positive cells. H1 cells were cultured in CM or CM + BMP4 (100 ng/ml) for seven days. Before termination of the culture, the cells were treated with brefeldin A. The cells were individualized, fixed, and followed by fluorescence-activated cell sorting analysis with the mouse anti-human CG-β primary antibody and fluorescein-labeled rabbit anti-mouse IgG secondary antibody. hCG, human chorionic gonadotropin.

tained with mouse anti-human CG-β antibody (Abcam, Cambridge, UK) at 1:100 dilution and fluorescein-labeled rabbit anti-mouse IgG antibody (Pierce, Rockford, IL) at 1:200. The cells were finally stained for the nuclei with Hoechst 33342 (Sigma) and photographed under phase and epifluorescence microscopy.

DNA microarray. H1 cells were treated in pairs with or without 100 ng/ml BMP4. Each pair of the cell samples was harvested at the indicated time points during the treatment. This was followed by RNA extraction and amplification[30] and microarray analysis on DNA chips containing 43,000 cDNA clones, which represented about 30,000 unique genes[31]. Areas of the array with obvious blemishes were manually flagged and excluded from subsequent analysis. All nonflagged array elements for which the fluorescent intensity in each channel was greater than 1.5 times the local background were considered well measured. Genes for which fewer than 70% of measurements across all the samples in this study met this standard were excluded from further analysis. We selected genes for further analysis with expression concentrations that differed by at least threefold in at least one sample. The results were visualized and analyzed with TreeView software (http://rana.lbl.gov).

Immunoassays of placental hormones in the culture medium. H1 cells were treated as above. The media conditioned on the cells were collected daily from days 3–8 and tested for CG-β using the AxSYM Total hCG-β kit (Abbott, Lake Forest, IL) and for estradiol[32] and progesterone[33] concentrations.

Flow cytometry. H1 cells were cultured in CM with or without 100 ng/ml BMP4 for seven days. Before harvest, the cells were treated with brefeldin A, individualized by treatment with trypsin/EDTA solution, fixed in 2% paraformaldehyde as above, and permeabilized by suspension in PBS containing 0.1% Triton X-100. The cells were filtered through a 40-μm mesh. Then 100 μl of the cell suspension containing 5×10^5 cells per tube were added to both a test tube and a control tube; 1 μl of mouse anti-human CG-β antibody (5 mg/ml) (Abcam) was added to the test tube, and 5 μl of mouse IgG (1 mg/ml) (Sigma) were added to the control tube. The tubes were briefly vortexed to mix and incubated for 30 minutes on ice; 1 μl fluorescein-labeled rabbit anti-mouse IgG antibody (Pierce) was then added, and the tubes were incubated for another 30 minutes on ice. The cells were washed twice and finally suspended in 0.3 ml of fluorescence-activated cell sorting buffer (calcium- and magnesium-free PBS + 2% fetal bovine serum + 0.1% sodium azide) for flow cytometry. The samples were analyzed on a FACSCalibur flow cytometer (Becton Dickson, San Jose, CA) using the Cellquest acquisition and analysis software (Becton Dickson). A total of 10,000 events were acquired, and analysis was restricted to live events based on light scatter properties. The fluorescein signal was collected through a 530/30 band pass filter, and the mean fluorescences for both the IgG control (data not shown) and the test samples were determined. All data were normalized by dividing the test mean by the control mean.

URLs. For time-lapse films, see http://genome-www.stanford.edu/es-cell/index.shtml.

Note: Supplementary information is available on the Nature Biotechnology website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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